

**An Analysis of LCAT in Regards to Egress of
*Toxoplasma gondii***

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Microbiology and Immunology)
in the University of Michigan
2018

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DEDICATION

This dissertation is the culmination of a long journey that quite likely would not have ever been completed without the help and support of many people. First, my labmates. Every person I have had the pleasure of working with in the Carruthers lab has provided invaluable support and oftentimes physical help at the bench. Mae, Bigbean, Ou, Olivia, and Geetha—thank you all so much for making it easy to keep coming in to lab. I don't know what I would have done if it weren't for you all, there with me every day. No matter where the future takes me I will always cherish the time spent working with you. To my family, for your love and support that never ever wavered, it has been difficult being so far away from you Mom and Dad (and Ashley, Mark, Andrea, Aaron, Annabella, Amelia, Arden, and Alicia), thank you for always believing in me. To my other family: Kyle, Andy, Drew, Hoppy, Christina, and Dan—thank you for never changing. I have seen very little of you while working on this dissertation, but the cherished few moments we have every year make me feel like I never left. Para hāgu Sophina, I guinaiyan kurason hu, ginen hāgu na hu hago' karerā-hu; thank you for keeping me in check.

ACKNOWLEDGEMENTS

The work described in the following pages was made possible through the contribution of many individuals. First, my thesis advisor Vern Carruthers, for constant mentoring. Countless hours of lab meetings, project updates, journal clubs, presentations, conference calls, and scientific meetings in which no detail was too small and all ideas were explored inside-out, front-back, top-bottom have slowly transformed me into a functioning (mostly) scientist. My thesis committee of Mary O’Riordan, Joel Swanson, Sarah Veatch and Jim Shayman have given me great suggestions along the way. It was a joy to have so many experts from different fields all come together and help steer my project in directions that I would have never explored if not for their expertise and guidance. I would like to thank Zhicheng Dou for suggesting that I try out a new method of cloning, Gibson assembly, that literally pushed my cloning projects months ahead of schedule. I would like to especially thank the members of the Shayman lab, particularly Bob Kelly, and Vania Hinkovska-Galcheva for help with PLA2 activity-based assays. More special thanks are in order to Joel Swanson and Sarah Veatch again for helpful guidance in the complex analysis of the calcium signaling experiments. The Microbiology and Immunology Department at the University of Michigan Medical School has been a wealth of knowledge for young trainees, and

particular thanks to Heidi Thompson for ensuring we were all taken care of and to Margaret Allen for help submitting and administrating grants and funding. Additional thanks are in order to our Italian colleague, Manlio Di Cristina, for creation of the Crispr-CAS9-Ble plasmid which has been a great improvement to the existing technology, to say nothing of his unabashed sense of humor.

This work was supported by the Molecular Mechanisms of Microbial Pathogenesis (MMMP) training grant (T32AI007528 to AJS), a predoctoral fellowship from the American Heart Association (AHA, 15PRE25720010 to AJS), and an operating grant from the National Institutes of Health (NIH, R01AI046675 to VBC).

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Abstract

Egress is a crucial phase of the *Toxoplasma gondii* intracellular lytic cycle. This is a process that drives inflammation and is strongly associated with the pathogenesis observed during toxoplasmosis. Despite the link between this process and virulence, little is known about egress on a mechanistic or descriptive level. Previously published work suggested that the parasite utilizes a phospholipase, lecithin-cholesterol acyltransferase (LCAT), for contributions to parasite growth, virulence, and egress. LCAT is secreted from the parasite's dense granules, but unlike other LCAT orthologues, this enzyme is proteolytically processed into two fragments. This processing is predicted to occur within a unique 140 amino acid insertion found inside LCAT's conserved catalytic domain. Here we present evidence from several independent mutant parasite lines confirming a role for LCAT in efficient egress, although no defects in growth or virulence were apparent. We also show via genetic complementation that the catalytic activity of LCAT is required for its role in parasite egress. Additionally, a genetically encoded calcium sensor revealed a potential delay in calcium signaling during egress in parasites lacking LCAT. This work solidifies the contribution of LCAT to egress of *T. gondii* tachyzoites and identifies a new connection in the calcium signaling network.

Chapter 1

Introduction

1.1 The apicomplexans

Found within the eukaryotic tree of life, branching off just before the divergence of plants and animals, are a small clade of unicellular pathogens belonging to the phylum Apicomplexa. This taxonomic group is where a number of clinically and agriculturally relevant microbes such as *Plasmodium falciparum* (malaria), *Cryptosporidium spp.* (cryptosporidiosis), *Babesia spp.* (babesiosis), and *Toxoplasma gondii* (toxoplasmosis) reside. These eukaryotic pathogens are named as such because of a common apically located plastid-like organelle, and share a number of important features like the use of a powerful actin-based motility system, a complex set of apical secretory organelles: the micronemes, rhoptries, and dense granules, and are all obligate intracellular parasites. Another hallmark of this phylum is its lytic intracellular lifecycle, which consists of parasite invasion into a host cell, replication of the parasite, and ultimately a dynamic egress event that leads to the cytolytic death of the parasitized host cell.

1.2 Infection by the tachyzoite

Although many stages of the parasite are found within a complete lifecycle, the most commonly studied forms of *T. gondii* in the laboratory are the tachyzoites. This

stage is responsible for the associated disease, toxoplasmosis. The term tachyzoite comes from Greek (tachos = speed) and refers to the high metabolism and reproductive rate seen in this form of the parasite. The infection begins with host cell invasion as the parasite uses adhesion molecules on its surface to attach to a host cell and correctly orient its apical end towards the target cell ^{1, 2}. Following attachment the tachyzoite secretes a number of proteins from the apical microneme and rhoptry organelles to form an invasion complex called the “moving junction” ³. This complex serves as an anchor point on the host cell which is coupled to the parasite’s underlying actin-myosin based motor system. As this complex is trafficked to the posterior end of the tachyzoite, the parasite effectively “pulls” itself into the host cell, invaginating the host plasma membrane along the way. The invasion process is completed as the host plasma membrane (HPM) is pinched off behind the now intracellular parasite, forming the parasitophorous vacuolar membrane (PVM) ^{4, 5}. Once within the parasitophorous vacuole (PV), additional proteins are secreted from a third set of secretory organelles called dense granules; straightforwardly named as such because of their dense appearance in electron micrographs. These GRA proteins are largely unexplored, and a distinguishing feature of identified GRA proteins is their lack of homology to proteins of known function. However, one prescribed role for GRA proteins is in the modification of the replicative niche and the PVM to optimize parasite growth and nutrient acquisition ^{6, 7}. Following invasion and replication, the intracellular lytic cycle is completed during egress, as the parasite escape the host cell confines and begin the cycle anew. This process shares many of the same aspects of invasion, as adhesins and effector

proteins are secreted from the micronemes, and the parasite's motor system becomes active once again.

1.3 Egress of *Toxoplasma gondii*

Intracellular infection is a strategy with a number of risks and rewards. While intracellular, *Toxoplasma* is surrounded by a wealth of host nutrients to support its growth and is shielded from the host immune system; however, in order for the infection to spread to a neighboring cell or to a new host organism the parasite must escape its infected host cell in a process called egress. Historically, egress was thought of as a simple passive event in which a nutrient exhausted host cell was “popped” from within due to the increasing mechanical burden of the rapidly expanding PV. The escaping parasites must breach multiple barriers including the parasitophorous vacuolar membrane, the host plasma membrane, host endomembranous organelles (e.g. mitochondria, endoplasmic reticulum), and host cytoskeletal structures. Recently we have come to appreciate this event is an active process in which the parasite utilizes a number of tools.

1.4 Host contributions to egress

In a twist that is not unsurprising given the level of control many parasites impose onto their hosts; new evidence suggests that *Toxoplasma* is able to hijack host proteases to facilitate its own release. Initial observations were made showing a requirement of host calpain, a calcium-dependent protease that degrades cytoskeletal structures. The primary experiments illustrated a role for this enzyme in egress of *Plasmodium falciparum*. Subsequent work showed that when *Toxoplasma* infected

murine cells lacking calpain 1 (siRNA knockdown or mutant cell lines), egress was delayed in comparison to infection of wild type cells⁸. A follow up study further explored this phenotype and found a role for a host G-protein coupled receptor (GPCR) at the head of a calcium signaling cascade involved in egress of both *T. gondii* and *P. falciparum*. Signaling through the GPCR led to activation of a phospholipase C (PLC) and protein kinase C (PKC). PKC dependent phosphorylation of α -adducin results in the destabilization of host cytoskeleton, followed by a calcium influx and finally, activation of host calpain-1⁹. However, it needs to be pointed out that evidence for a parasite-induced mechanism of GPCR activation was based solely on a loose association: the particular GPCR in question (G α q subunit), can be activated by alpha ketoglutarate and succinate—both bi-products of the *T. gondii* TCA cycle. No direct role for these parasite-derived molecules was shown; therefore these findings should be interpreted cautiously in the absence of additional data.

1.5 Barriers to egress

As previously mentioned, there are numerous lipid bilayers that *Toxoplasma* must circumvent in order to egress. As of yet only a single protein, perforin-like protein 1 (PLP1), has been attributed to this process. PLP1 is secreted from the micronemes, most likely immediately prior to egress, and functions similarly to other pathogenic pore-forming proteins by oligomerizing on target membranes before insertion as a large multimeric pore^{10, 11}. While genetic data clearly shows that PLP1 is both necessary and sufficient for permeabilization of the PVM, it is not yet known if PLP1 is sufficient for lysis of host membranes, functions cooperatively or in synergy with other egress factors,

and/or serves as a conduit for release of additional lytic factors or exchange of signaling molecules between the host and pathogen. More recent work has illustrated similar roles in egress for PLP1 homologues in various stages of the *Plasmodium spp.* lifecycle as well.

In the *Plasmodium* egress framework, it is clear that secreted proteases help facilitate egress, as initial experiments showed that E64, an inhibitor of cysteine proteases, efficiently blocked parasite egress¹², and shortly thereafter was confirmed genetically as a number of proteases were implicated in egress of the malaria parasite¹³⁻¹⁶. As of yet there have been no mechanistic proposals for how these molecules contribute to parasites egress, but unpublished data from our lab show egress defects in two independent knockouts in *T. gondii* proteases, SUB1 and TLN4—although to a much lesser degree than the PLP1 knockout.

1.6 The micronemes

While much of the underlying mechanics of egress remain an enigma for all apicomplexan parasites, data examining this topic in *Toxoplasma* and *Plasmodium spp.* make it very clear that microneme secretion is vitally important to this process. In addition to secretion of PLP1, microneme secretion is crucial for parasite motility. The positive influence of motility on egress has long been considered important for efficient egress, yet not essential as chemically paralyzed *Toxoplasma* tachyzoites were observed rupturing the infected host cell¹⁷. However more recent work on knockdowns of actin and associated motor proteins in *Toxoplasma* have demonstrated that

genetically paralyzed parasites are able to invade but are lethally impaired in their ability to egress¹⁸.

Perhaps the most telling evidence for the importance of microneme secretion comes from a chemical mutagenesis screen to generate a pool of temperature sensitive mutants; among these mutants they identified a clone that was unable to secrete microneme proteins at the restrictive temperature and was completely unable to egress at 40°C. Using whole genome sequencing of the mutant the phenotype was traced to a protein called DOC2.1¹⁹. This protein contains double C2 domains, a feature shared with proteins that facilitate membrane association in response to calcium. The proposed function of DOC2.1 is that it orchestrates membrane fusion and exocytosis of micronemes, perhaps in conjunction with other proteins such as SNAREs, in response to calcium.

1.7 Signals for egress

Parasite intracellular calcium has been recognized for quite some time as a key signal for microneme discharge²⁰, and accordingly this event has a prominent regulatory role in parasite invasion and egress. While these observations were initially made with chemical treatments that liberated stored calcium (calcium ionophores, thapsigargin), the explosion of genomic data in recent years has revealed an apicomplexan genome rich with proteins containing known calcium-binding domains. Pharmacological studies in *Toxoplasma* have shown the presence of a nifedipine-sensitive calcium channel²¹ as well as PLC enzymes that generate IP₃²². Importantly, the parasite itself responds to IP₃ treatment with calcium release²³. In addition to IP₃,

another molecule known to play a role in calcium signal amplification is cyclic ADP Ribose (cADPR), and again *T. gondii* undergoes a calcium response upon treatment with this molecule ²⁴. While pharmacological data suggests the existence of these pathways, no IP₃ receptors or cADPR receptors have been identified, and there are no obvious homologues to these proteins in the Apicomplexan genome.

While calcium has long been implicated in microneme secretion and egress, it wasn't until recently that some of the underlying factors have been reported. The Sibley group identified a calcium-dependent protein kinase (CDPK1) that acts downstream of calcium as a messenger and is essential for secretion of the micronemes ²⁵. Shortly thereafter work expanding on this family was published as three independent groups detailed the role of another calcium-dependent protein kinase, CDPK3 ²⁶⁻²⁸. All three groups found that CDPK3 was essential for microneme secretion while the parasite was intracellular (relevant to egress), but dispensable for microneme exocytosis while extracellular (relevant to invasion); and that CDPK3 is capable of sensing a drop in environmental K⁺ concentrations in the host cytosol ²⁶. Sensing of environmental K⁺ brings up an important point in what the natural signal is for parasite egress. Historically, study of egress in the lab has relied on the artificial induction of egress by forcing the parasite to release calcium from intracellular stores. However, multiple groups have published that loss of host cell K⁺ can trigger this event ^{29, 30}, and have postulated that *in vivo* potassium loss may be a signal that an infected host cell is under immune attack. In an event called externally triggered egress (ETE), parasites that are infecting macrophages or monocytes that come under immune attack through perforin or death ligand (Fas) signaling will initiate egress; ^{31, 32}. Interestingly the consequences of ETE

are unclear; on one hand ETE seems to be a survival mechanism for the parasite, serving as a convenient way to escape from immune clearance. On the other hand, in a process called haven disruption, parasites that underwent rapid cycles of egress and reinfection were selectively eliminated *in vivo* ³¹.

In the absence of immune pressure, the parasite may be able to use endogenous clues to initiate calcium-dependent egress through the hormone abscisic acid (ABA) ³³. ABA levels within the parasite are relatively low except in more mature vacuoles prior to egress and are thought to act by inducing the production of the previously mentioned cADPR.

1.8 A new player in egress

Despite the important role of egress in the tachyzoites intracellular lifestyle, this process remains largely enigmatic as only a single secreted factor, PLP1 has been mechanistically described ¹⁰. This notable gap drove a search for additional secreted factors, which led us to a parasite-derived phospholipase: annotated within the database (ToxoDB) as lecithin-cholesterol acyltransferase (LCAT). A *Plasmodium* orthologue of LCAT was shown to be an active phospholipase that plays a pivotal role in the murine model of malaria³⁴. However, this protein does not seem to be necessary for *Plasmodium* sporozoite egress, but instead is surface localized and involved in cell traversal—a cell migratory process with many analogies to *T. gondii* egress.

Consistent with our hypothesis we showed that Δ /cat tachyzoites are delayed in their ability to egress from infected fibroblasts *in vitro* ³⁵. While this discovery represents the first description of a phospholipase utilized during egress by an apicomplexan

parasite, the use of lytic molecules such as pore-forming toxins (PFTs) and phospholipases shared among many clinical intracellular pathogens³⁶. The model system in this regard has been *Listeria monocytogenes*, which uses the pore-forming toxin LLO, and two phospholipase C (PLC) enzymes, PI-PLC and PC-PLC to escape limiting vacuoles after internalization into the host cell³⁷. Despite clear thematic similarities, there are at least two important differences between *Toxoplasma* LCAT and *L. monocytogenes* PLCs. First, *Listeria* PLCs attack the C1 position, generating the intracellular signaling molecules DAG and IP₃. In contrast LCAT displays phospholipase A2 (PLA2) activity³⁵, which generates lysophospholipid and releases a free fatty acid from the C2 position—neither of which have any implicated roles in intracellular signaling. However, lysophospholipid products from phospholipase reactions have been shown to have cytolytic activity³⁸. Second, *L. monocytogenes* escapes its vacuole shortly after invasion of the host cell, while *Toxoplasma* presumably secretes LCAT shortly after invasion and replicates for up to 60 h *in vitro* before initiating egress. This suggests there are intrinsic differences in how the activities of these two molecules are regulated. One potential method the parasite may employ to regulate the activity of egress effector proteins is by modulating a pH drop within the parasitophorous vacuole³⁹. Importantly, in studies using the human orthologue of LCAT, a drop in pH enhances the enzyme's activity by promoting membrane binding through an electrostatic interaction with negatively charged lipids⁴⁰.

In addition to a conserved PLA₂ domain, *Tg*LCAT also possesses a lipase motif, AHSLG, that is characteristic of LPLA2 enzymes and has a conserved catalytic triad of SDH. *Tg*LCAT is secreted from the parasite's dense granules, but in contrast to known

members of the PLA₂ family, the parasite protein is cleaved into two fragments post-secretion³⁵. To gain a perspective on how the parasite utilizes this protein during the tachyzoite stage of infection, we examined the phenotype of parasite strains lacking the *lcat* coding sequence in the acute stage of the infection, the tachyzoite. Our results suggest that *Tg*LCAT plays a positive role in facilitating parasite egress from the host cell, and this phenotype is potentially modulated by altered calcium signaling within the tachyzoite. We were not able to observe any defects in the ability of these knockout parasites to replicate or cause disease in the mouse model of infection, suggesting that LCAT does not contribute to growth or virulence.

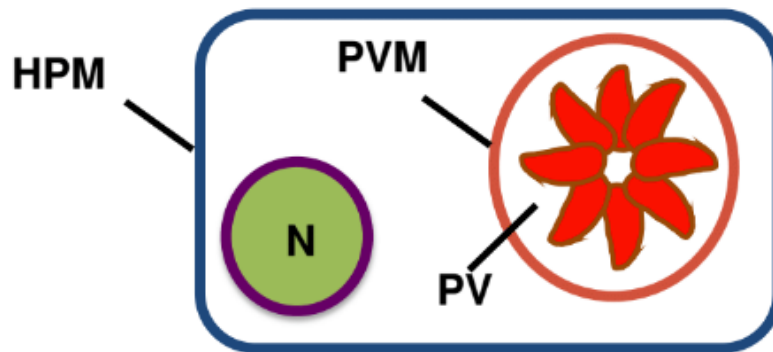


Figure 1-1. Intracellular schematic of the tachyzoite. Schematic illustrating host cell infection by *T. gondii* and relevant structures to egress. HPM = host plasma membrane; PV = parasitophorous vacuole; PVM = parasitophorous vacuolar membrane

Chapter 2

***Toxoplasma gondii* LCAT primarily contributes to tachyzoite egress**

2.1 Introduction

Toxoplasma gondii is an obligate intracellular parasite that infects a wide range of host animals, including humans. This parasite is estimated to infect up to 1 in 3 people worldwide ⁴¹, who are presumed to harbor the infection for the remainder of their lifetime. *Toxoplasma* commonly infects humans congenitally, through the ingestion of oocysts shed in the feces of infected felines, or from consumption of undercooked meat that contains tissue cysts ⁴². For *T. gondii*, and other intracellular pathogens, egress represents a crucial step of the lifecycle, which must occur in order for the parasite to infect new host cells. With this in mind it is not surprising that intracellular microbes have evolved numerous approaches and strategies to complete this task ³⁶. These different approaches have vastly different impacts on the host and the damage-response to infection ⁴³. One such strategy utilized by protozoan parasites and extensively within *Toxoplasma*'s apicomplexan phylum is cytolytic egress. This event in particular results in massive release of inflammatory signals from cell death and tissue destruction. The resulting pyrogenic immune response is a hallmark of both

toxoplasmosis and malaria, caused by the related apicomplexan *Plasmodium falciparum*. While infection of a healthy adult is typically benign, toxoplasmosis can be disastrous among immunocompromised patients or those infected congenitally. Common manifestations of pathogenesis seen include potentially fatal toxoplasmic encephalitis and retinal destruction, frequently observed among congenitally-infected individuals ⁴⁴⁻⁴⁶. Indeed, the importance of a controlling host immune response is illustrated while examining the devastating affect of toxoplasmosis on the mortality of HIV-AIDS patients in the pre-antiretroviral (HAART) era ⁴⁷⁻⁴⁹, an observation that underscores the need for robust monitoring of tissue transplant and/or chemotherapy patients today.

At the center of the pathogenesis seen during toxoplasmosis, is the lytic cycle of *Toxoplasma*. This cycle begins with the active invasion of the parasite into a new host cell, and the formation of an intracellular replicative niche called the parasitophorous vacuole (PV), formed from the host plasma membrane during invasion ⁵⁰. After replication within the sequestered PV, the tachyzoites actively egress from the host cell and begin the cycle anew by invading nearby host cells. In many aspects invasion and egress are similar events and rely on secretion of the parasite's micronemes and parasite motility, both processes dependent on activation of intracellular calcium signaling pathways ⁵¹. Egress requires the breakdown of two notable membrane barriers: the parasitophorous vacuolar membrane (PVM) and the host plasma membrane (HPM). However, outside of the microneme derived perforin-like protein 1 (PLP1) which permeabilizes the PVM ¹⁰ effector molecules directly involved in this process have not been thoroughly defined.

Lecithin-cholesterol acyl transferase (LCAT) was originally described in *Plasmodium berghei* as a phospholipase named “PL”, in a screen to identify sporozoite proteins involved in the establishment of the infection ³⁴. More recently this phospholipase has been tied to permeabilization of the PVM during *Plasmodium* merozoite egress from hepatocytes ⁵². Since then, additional work has been published focusing on the *Toxoplasma* ortholog LCAT. A mutant strain lacking LCAT showed slower growth based on fewer parasites per PV and smaller plaque area, a defect in egress, and a notable loss in virulence manifested by 70% survival of mice infected with mutant strains versus 0% survival of those infected with the parental strain ³⁵.

To understand in greater detail the secreted effectors that *Toxoplasma gondii* utilizes during egress, we have focused our attention on TgLCAT, hereafter referred to simply as “LCAT”. During the course of this study, we consistently observed that parasites lacking LCAT were unable to complete the egress event as efficiently as their wildtype counterparts. However, unlike the previously described LCAT mutant ³⁵, the new strains show normal parasite replication *in vitro* and no loss of virulence in mice. Interestingly, we also establish a link between expression of LCAT and the kinetics of intracellular calcium signaling. This is based on Δ /*cat* parasites displaying a lag in calcium signaling in response to zaprinast treatment as measured via a genetically encoded calcium sensor. These findings establish a new connection between a secreted phospholipase and calcium signaling in *Toxoplasma*.

2.2 Results

2.2.1 Genetic removal and complementation of LCAT

Previous work regarding LCAT focused on parasites that were genetically ablated in the wild-type RH background ³⁵. This approach led to the generation of a knockout line that might contain additional genetic disruptions and a complemented strain that substantially overexpressed active LCAT. Also, the previous study did not include a complementation strategy with a catalytically inert mutant. We reasoned that these issues would complicate downstream analyses and that, accurately assessing the function of LCAT would require remaking the knockout in the RH $\Delta ku80$ line, which allows more precise gene deletion and complementation ⁵³. The LCAT locus (EuPathDB: TGME49_272420) was modified by double homologous integration to replace the LCAT coding region with the dihydrofolate reductase (*dhfr*) selectable marker. After transfection of a linear knockout construct (**Figure 2-1A**) constructed by fusion PCR and pyrimethamine selection, the absence of LCAT was confirmed genetically via PCR amplification of the coding sequence and at the protein level as seen by western blot (**Figure 2-1C,D**). This RH $\Delta ku80\Delta lcat$ strain was then complemented with C-terminally HA tagged WT ($\Delta lcat$ LCAT-HA) and a mutant allele ($\Delta lcat$ LCAT*-HA), which harbors an S332A point mutation predicted to destroy lytic activity from the active site serine. These constructs were integrated into the “empty” $\Delta ku80$ locus by double homologous recombination (**Figure 2-1B**) and incorporation into the genome was confirmed by PCR detection of the shortened cDNA within the *ku80* locus and by restoration of protein expression, albeit slightly shifted in size due to the addition of the epitope tag (**Figure 2-1C,D**).

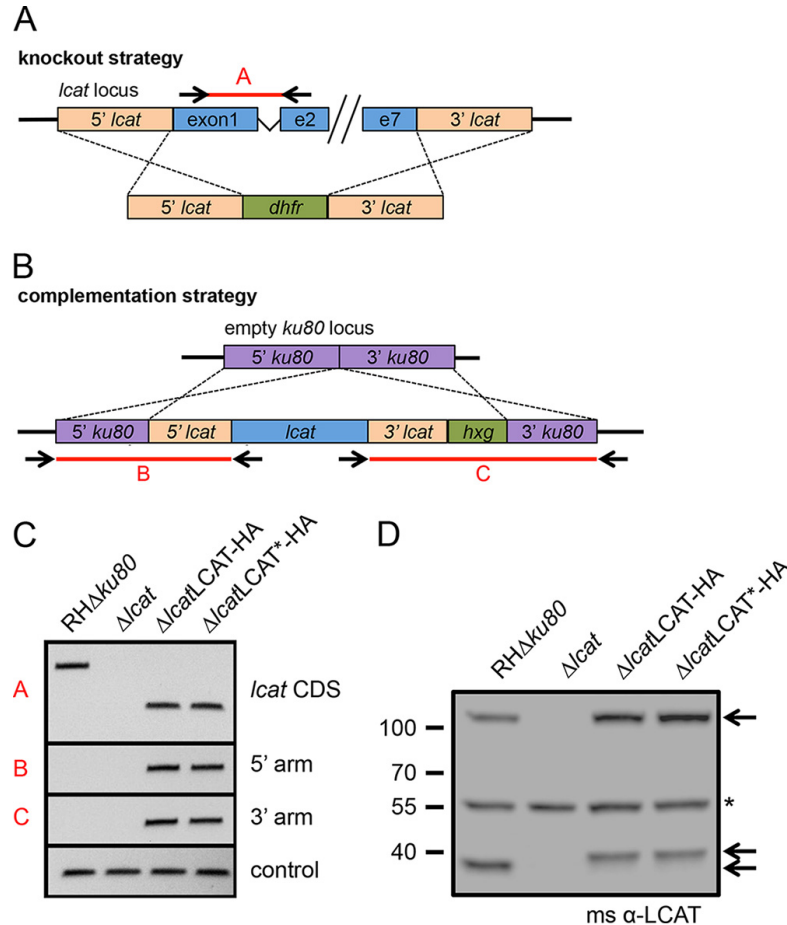


Figure 2-1 Generation of $\Delta lcat$ and $\Delta lcat$ complemented lines. (A) Schematic representation of the linear knockout construct and generation of the LCAT knockout by double homologous integration of *dhfr* at the *lcat* locus. e2, exon 2. (B) Schematic representation of the complementation of the $\Delta lcat$ strain, showing the *lcat* cDNA under direction of its endogenous 5' and 3' untranslated regions (UTRs), being driven to integrate into the "empty" *ku80* locus by double homologous recombination. (C) PCR showing genetic confirmation of the knockout and complemented strains by amplification of the coding sequence (CDS) (primer pair A), and detection of the 5' and 3' arms of the complementation construct within the *ku80* locus (primer pairs B and C, respectively). (D) Western blot confirming the loss of and restoration of LCAT expression in the knockout and complemented strains, respectively. The positions of molecular weight markers (in kDa) are indicated to the left of the blot. The arrows to the right of the blot indicate specific full-length and proteolytically processed bands (slightly shifted in the complemented strains, due to the addition of HA epitope tag).

2.2.2 LCAT mutants show normal plaque formation and replication

We next performed plaque assays to test the LCAT deficient parasites for any broad defects through successive lytic cycles. Confluent monolayers of human foreskin fibroblasts (HFFs) were infected, and the parasites were allowed to replicate undisturbed for 7 days before fixation and staining of the monolayer (**Figure 2-2A**). Quantification of the resulting plaque sizes showed no difference between parental, knockout, or either of the complemented strains (**Figure 2-2B**). These results indicate the absence of LCAT does not affect the ability of tachyzoites to efficiently progress through the lytic cycle *in vitro*. While the results were not suggestive of a growth defect, we shifted to an intracellular replication assay to more precisely examine the rate of parasite division between strains. Parasite replication was directly measured by infecting confluent monolayers of HFFs and allowing replication to occur for either 16 or 32 hours before fixation and manual quantification of parasites per vacuole via fluorescence microscopy. No differences in the replication rate of Δcat parasites compared to parental or complemented strains were found (**Figure 2-2C,D**).

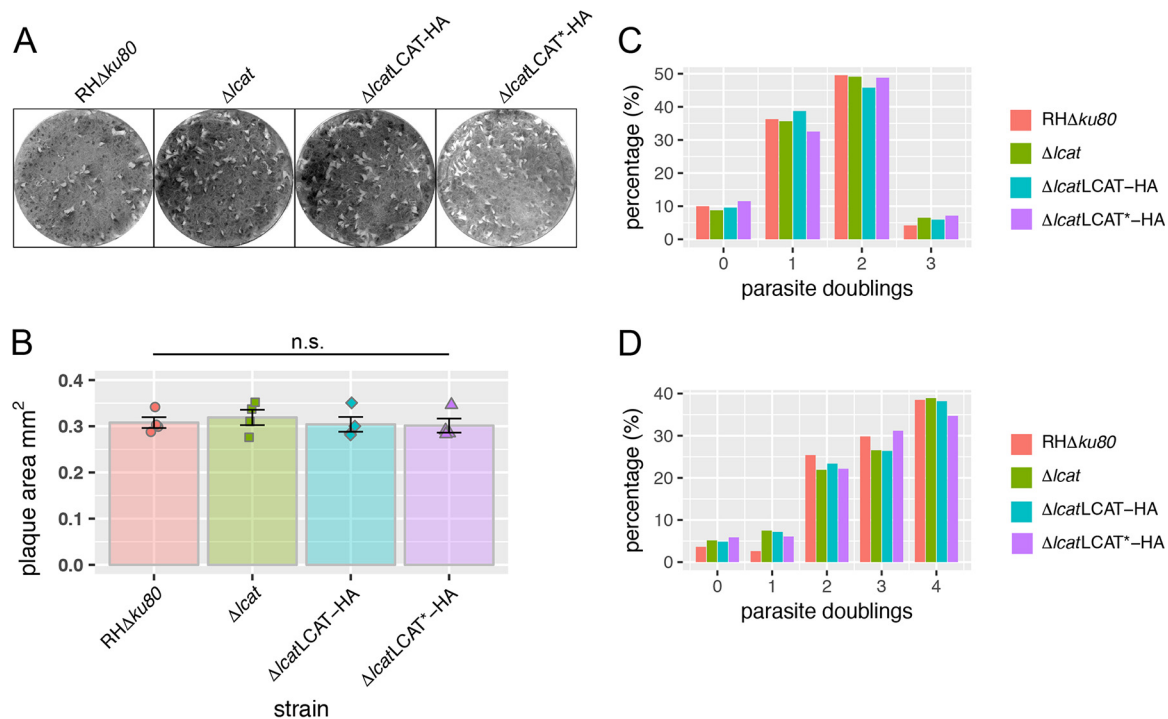


Figure 2-2. LCAT does not play a role in growth or replication.

(A) LCAT knockout and complemented strains form normal plaques over a 7-day infection of HFFs. (B) Quantification of the plaque area via ImageJ showed no significant differences between parental, knockout, or complemented strains. Data shown are means standard errors of the means (SEM) (error bars) from three or four biological replicates. The values were not significantly different (n.s.). (C and D) Quantification of parasite replication by counting parasites per vacuole at 16 h (C) or 32 h (D). Data represent pooled data from three biological replicates, each with at least 250 vacuoles per strain counted. No significant differences were found using Pearson's chi-squared test.

2.2.3 LCAT deficient parasites display normal virulence in the murine model

We next sought to measure virulence of the new *Δlcat* strain in the acute model of murine infection. Due to the highly virulent nature of the type I strain background, we chose to use a low dose of 10 tachyzoites administered subcutaneously to BALB/c mice. By 10 days post infection infected mice were displaying signs of morbidity, and were euthanized by 10 – 15 days post infection (**Figure 2-3A**). No statistically significant

differences in survival were seen as mice infected with parental, knockout, or complemented strains succumbed to the infection with similar kinetics. To more thoroughly investigate a potential role for LCAT *in vivo*, we re-created the knockout in the wild-type RH background. LCAT deficient parasites were generated using the CRISPR-Cas9 system⁵⁴, by targeting the Cas9 nuclease via a single guide RNA to a region near the 5' end of *lcat* exon 1 (**Figure 2-3B**). After transfection of the plasmid containing the *lcat* sgRNA, we were able to isolate parasite clones that no longer expressed the LCAT protein, as confirmed by western blot shown for one such clone (**Figure 2-3C**). No difference in plaque area was observed between wild-type and RH Δ *lcat* tachyzoites, confirming that LCAT deficient parasites progress normally through the lytic cycles and our initial observations in the $\Delta ku80$ background (**Figure 2-3D**). In lieu of further quantification by counting parasites per vacuole as done previously, we chose to use an *in vitro* competition assay which, over serial passages, should be a more sensitive method of quantifying parasite growth. Briefly, RH Δ *lcat* parasites were transfected with a plasmid allowing for stable expression of GFP to allow for easy identification and differentiation of RH vs. RH Δ *lcat* parasites. Both parental and knockout parasites were inoculated into T25s of confluent HFFs, and after each lysis parasites were collected for quantification via fluorescence microscopy. The results of the *in vitro* competition assay mirror what was seen in previous growth assays, as there were no significant differences in relative abundance after serial co-culture (**Figure 2-3D**).

To measure virulence of the new RH Δ *lcat* strain we injected BALB/c mice subcutaneously with 10 or 50 tachyzoites of wild-type or two clones of RH Δ *lcat*. Again,

no statistically significant differences were elucidated between any of the strains (**Figure 2-3F,G**). This experiment was repeated in outbred mice, as we infected Swiss Webster mice with 50 tachyzoites of wild-type or a single clone of $RH\Delta/cat$, with very similar results being observed (**Figure 2-3H**). Collectively, after testing the LCAT knockout from multiple parasite strain backgrounds, in the context of multiple mouse strain backgrounds, our findings suggest that LCAT does not contribute to *Toxoplasma* virulence.

2.2.4 Parasites lacking LCAT display a consistent phenotype in impaired egress

We next examined to what extent the loss of LCAT expression affects parasite egress from the host cell. To determine this we infected HFF monolayers in 96-well plates with either WT (RH or $RH\Delta ku80$) or knockout ($RH\Delta/cat$ or $RH\Delta ku80\Delta/cat$) parasites. After 30 hours of replication the infected monolayers were then treated with zaprinast to pharmacologically induce egress. Zaprinast is a phosphodiesterase inhibitor that induces egress by activation of the parasite protein kinase G (PKG)²⁷. The culture supernatant was collected and assayed for lactate dehydrogenase (LDH) content as a function of egress (host membrane damage). The results showed ~40% and ~55% in zaprinast-induced LDH release by $RH\Delta/cat$ and

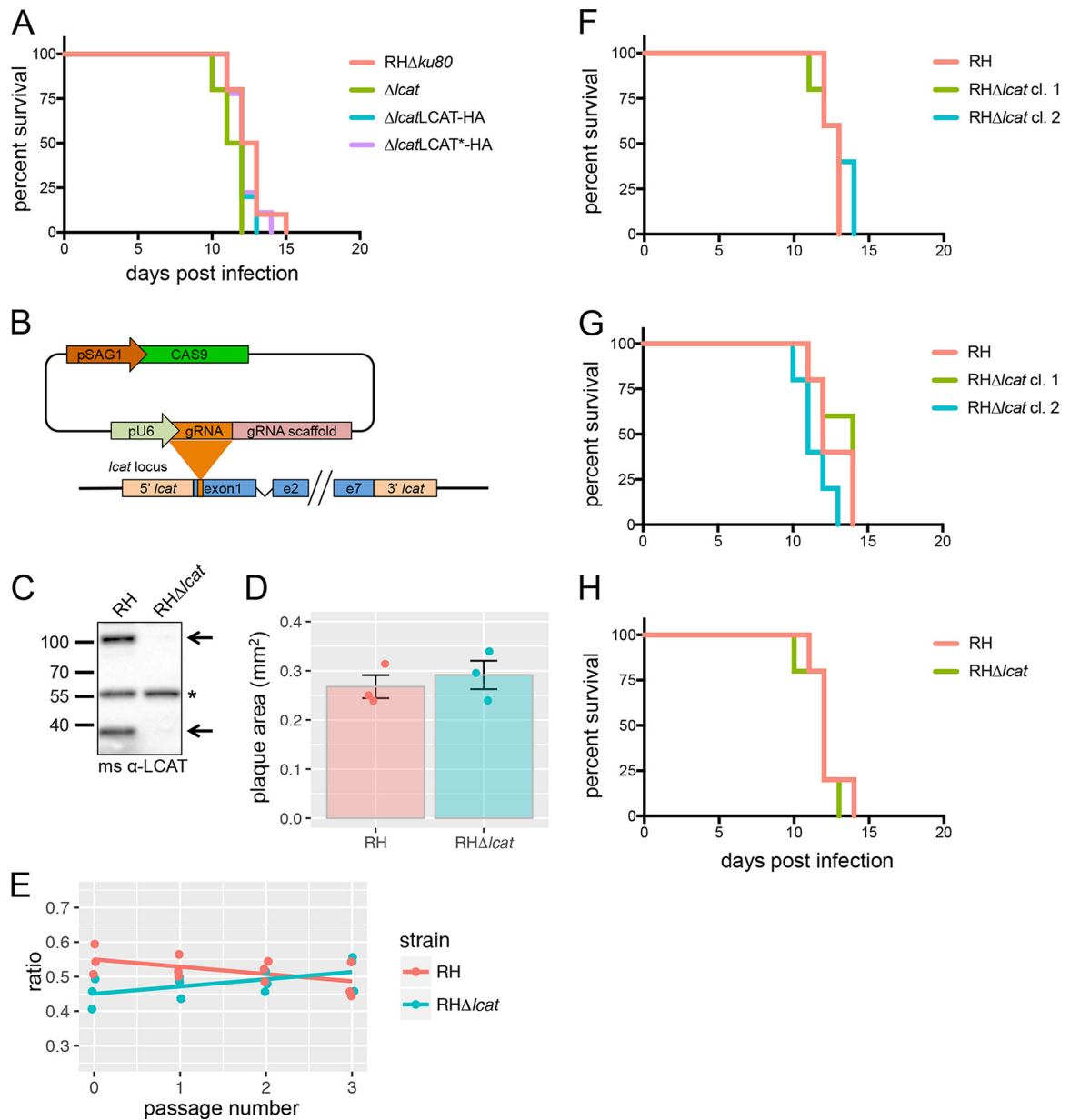


Figure 2-3 LCAT does not contribute to virulence in the murine model. (A) Survival data of infected mice. Female BALB/c mice were infected subcutaneously with 10 tachyzoites of the indicated strain, and survival was monitored over the following weeks. The data represent 10 mice per group pooled from two biological replicates. (B) Schematic illustrating the use of CRISPR-Cas9 for targeted disruption of the *lc Δ* coding sequence. A 20-bp sgRNA was used to direct the nuclease to the 5' region of exon 1. (C) Western blot confirming the loss of LCAT expression after CRISPR-Cas9-mediated disruption. The asterisk denotes a nonspecific band. (D) Quantification of plaque area in wild-type RH and RH Δ lc Δ . (E) Parasite growth as measured by an *in vitro* competition assay showing the relative abundance of RH and RH Δ lc Δ across three serial passages. (F) Survival of female BALB/c mice infected with 10 tachyzoites subcutaneously. (G) Survival of female BALB/c mice infected with 50 tachyzoites subcutaneously. Data in panels F and G are from 10 mice per group pooled from two biological replicates infected with wild-type or two separate clones of RH Δ lc Δ . (H) Survival of female outbred Swiss Webster mice infected with 10 tachyzoites subcutaneously. The data are from five mice in one experiment. No significant differences were observed in growth (Student's *t* test) or survival (Kaplan-Meier analysis).

RH Δ lcat Δ ku80 parasites, respectively (Figure 2-4A,B). This phenotype was partially, yet significantly, restored upon complementation of the knockout with the wildtype version of LCAT but not with the catalytically inert mutant (Fig. 4B). These findings confirm a role for LCAT in *Toxoplasma* egress, and newly identify a requirement for LCAT activity in this event.

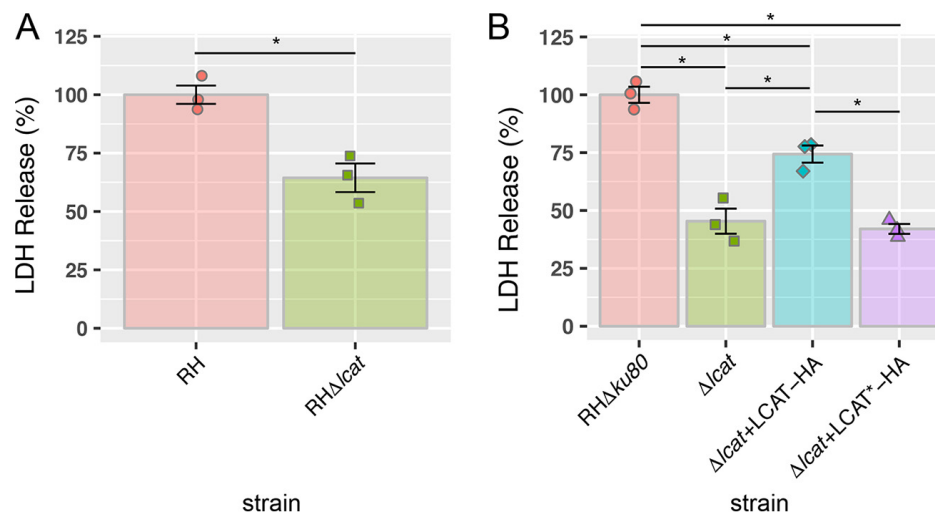


Figure 2-4. LCAT-deficient parasites are unable to efficiently egress from host cells. (A and B) Egress following 20 min of incubation with 57 M zaprinast. Lactate dehydrogenase release following induction was used as a measure for egress and normalized to wild-type RH (A) or RH Δ ku80 (B). Statistical significance was assigned by Student's *t* test, Data shown are means SEM (error bars) from three biological replicates each with three technical replicates. Values that are significantly different (*P* 0.05) by Student's *t* test are indicated by a bar and asterisk.

2.2.5 Parasites lacking LCAT have altered calcium signaling kinetics

To more thoroughly investigate the nature of the egress defect, we next sought to examine calcium signaling as this event has been well established as a central regulator of parasite motility and egress. To this end, *lcat* was again genetically ablated using CRISPR-Cas9, this time in a parasite line expressing a genetically encoded calcium sensor (GCaMP6f) (**Figure 2-5A**). This system allowed us to monitor calcium signaling in real time, as changes in intracellular calcium concentration during zaprinast-induced egress lead to a robust response of increased GCaMP fluorescence (**Figure 2-5B**). To analyze calcium signaling, HFF monolayers were infected for 30 hours prior to induction of egress by treatment with zaprinast. Calcium signaling was quantified as the time of maximum GCaMP intensity, as measured over the entire PV. We were able to detect a subtle difference in the timing of the peak signal (**Figure 2-5C**, upper panel), which was significantly exacerbated in the absence of extracellular calcium (**Figure 2-5C**, lower panel). While analyzing these data, we noted the resulting calcium traces (GCaMP intensity vs. time) could be binned into two types of response categories: monophasic and biphasic (**Figure 2-5D**). While the ratio of monophasic:biphasic response was roughly 1:1 for both wildtype and knockout strains, we chose to examine the kinetics of the biphasic response more closely by characterizing the elapsed time between the two peaks (**Figure 2-5D**). Once again we were able to detect a subtle phenotype that was significantly separated in the absence of extracellular calcium (**Figure 2-5E**). Further analysis of this phenotype led us to examine the biphasic vacuoles that successfully egressed during the 4-minute course of the experiment. After ranking egress as a percentage of the population over time (multiple pooled experiments), the findings

suggest the delay between these peaks observed in *GCaMP Δ cat* parasites contributes to a postponement of egress by these parasites (**Figure 2-5F**). Unfortunately, attempts to complement the phenotype generated data with a similar trend, but were not statistically significant. The *GCaMP Δ cat* strains were complemented using the same linearized complementation construct previously discussed (**Figure 2-1**), and although these new lines generated the expected egress phenotypes (**Figure 2-5G**), the calcium signaling phenotype did not (**Figure 2-5H**).

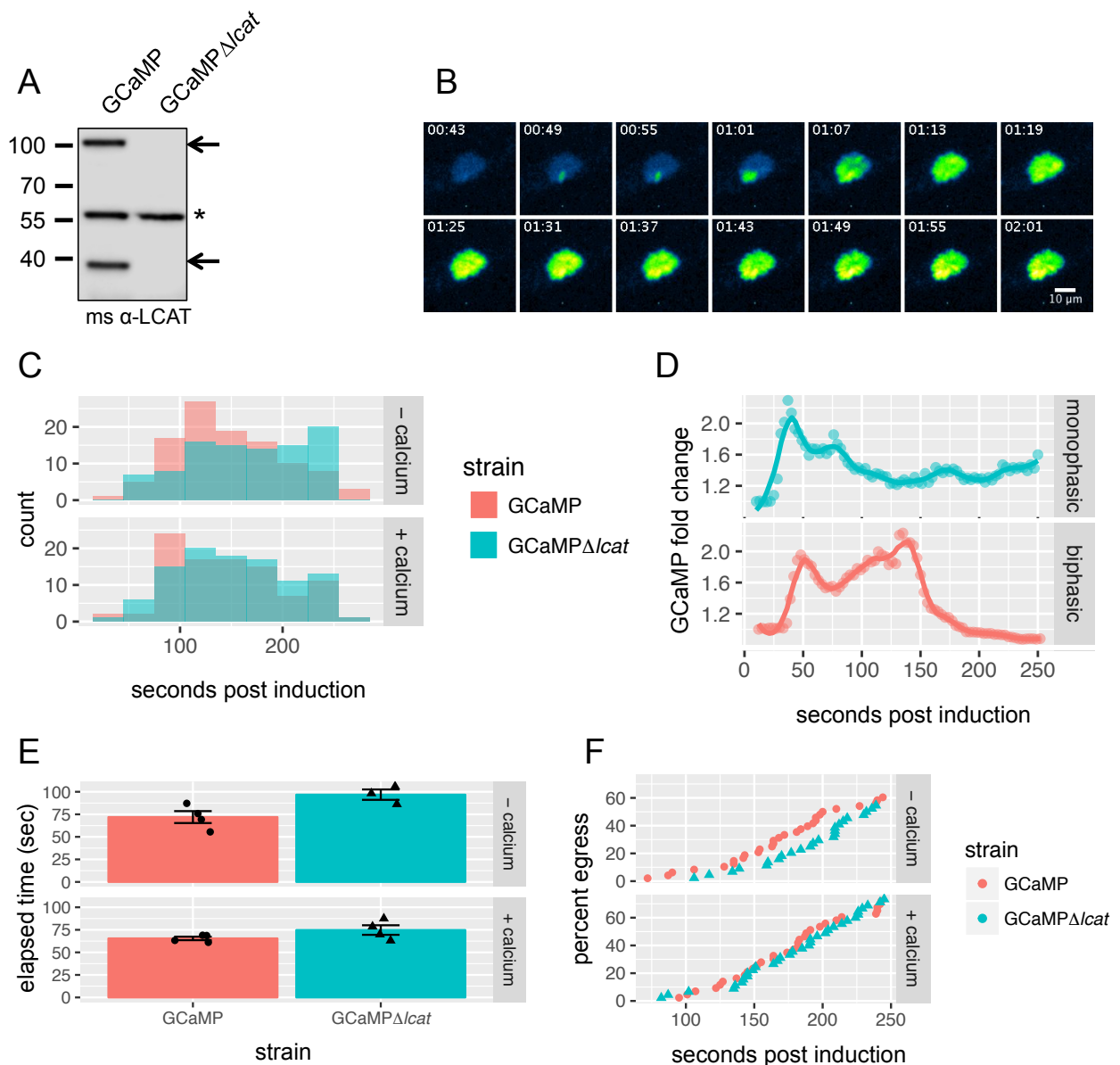


Figure 2-5. Parasites lacking LCAT have altered calcium signaling kinetics. (A) Western blot showing loss of LCAT expression in the GCaMP background following CRISPR-Cas9 disruption. (B) Example time-series of images showing a robust response in fluorescence intensity following treatment of GCaMP infected HFFs with 200 μ M zaprinast. (C) Distribution of the maximum GCaMP signal. Fluorescence intensity was tracked following treatment with zaprinast and the time of the maximum signal over the entire PV was extracted. The histograms represent pooled data from four independent experiments, significance was tested by Wilcoxon log-rank. $P < 0.05$, - calcium. Data are pooled from 4 biological replicates each with evaluation of 76 – 153 PVs per experiment; $N = 399$. (D) Two representative traces showing the monophasic and biphasic classes of responding vacuoles. (E) Duration between the initiation and potentiation peaks in vacuoles that respond in a biphasic manner. Data shown are means \pm s.e.m. from 3-4 biological replicates. (F) Egress kinetics among biphasic responding vacuoles. Pooled data from 4 independent experiments showing the percentage of egress over time in ranked order.

2.2.6. Altered Kinetics of PVM permeabilization in Δ /cat parasites

To further examine the defects seen in the *lcat* knockout strains, we performed assays to monitor the integrity of the PVM in wild type and *lcat* knockout strains. To accomplish this, we used the wild type GCaMP and GCaMP Δ /cat parasites previously described, and transiently transfected them with a construct encoding a soluble form of mCherry that is secreted into the PV (**Figure 2-6A**). Prior to egress while the PVM is intact, the mCherry signal is contained within the PV. Following treatment with zaprinast to stimulate egress and cytochalasin D to paralyze the parasites and halt motility, the intensity of this marker rapidly diminishes as mCherry diffuses out of the PV and into the host cytoplasm (**Figure 2-6B**). The inclusion of the GCaMP signal allowed us to use the maximum GFP intensity as a reference point, and compare the kinetics of when mCherry is released in the Δ /cat mutants. Median times of mCherry release between the two strains trended toward being different (**Figure 2-6C**) , but did not reach statistical significance due to small sample sizes and variability in the response times, especially for GCaMP parasites.

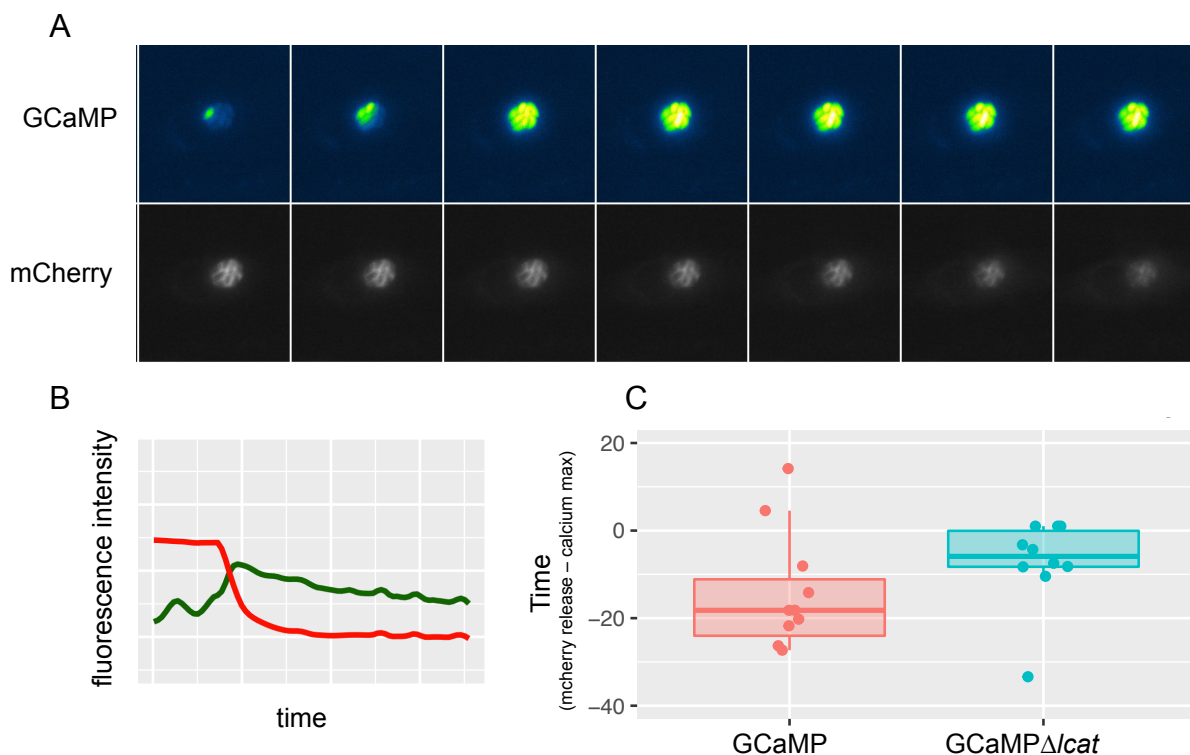


Figure 2-6. mCherry release from the PV in parasites lacking *Icat*.

(A) Series of images showing the increase in GCaMP intensity (top row) and the accompanying loss of mCherry signal from the PV as the PVM is ruptured (bottom row). (B) One representative trace illustrating what a typical calcium response and mCherry loss looks like. (C) Quantification of mCherry release from the PV in relationship to calcium response. mCherry loss was defined as the time point of the greatest change in mCherry intensity within the PV (ΔI), and was normalized to the time of maximum GCaMP intensity (I_{\max}). Data are representative of 2 independent experiments, no significance in the median distribution was found by Wilcoxon log-rank test.

2.3 Discussion

As obligate intracellular pathogens, egress from the infected host cell is an absolute requirement for apicomplexan parasites. So it comes as no surprise these parasites have evolved a multitude of specialized effector molecules to complete this task. While currently only one such protein has been mechanistically described in *Toxoplasma* (PLP1)^{10, 11, 39}, there have been suggested roles for a genetically duplicated secreted nucleoside triphosphate hydrolase⁵⁵ that could act in signaling or

as an effector. Host calpain proteases have also been implicated, which are thought to facilitate egress by selective degradation of the host cytoskeleton prior to egress^{8, 9}. The landscape of secreted egress effectors has become somewhat better defined in the *Plasmodium* field, with roles being ascribed for secreted proteases such as SUB1, members of the SERA family, and DPAP3^{15, 16, 56} in addition to several members of the perforin-like protein family⁵⁷⁻⁵⁹.

In 2005 a surface localized phospholipase, PbPL, was described in *Plasmodium berghei*, which facilitates sporozoite cell traversal³⁴, and was more recently shown to aid in merozoite egress from infected hepatocytes⁵². Subsequently the *Toxoplasma* orthologue LCAT was shown to have a similar role in egress of tachyzoites *in vitro*³⁵. In a continuation of that study we have found a consistent role for LCAT in parasite egress, but were not able to identify a supporting role in parasite replication or a noticeable role *in vivo*. One possible explanation is the original LCAT knockout was made in the RH strain, which can exhibit off target integration of knockout constructs. Replication and virulence phenotypes in the original LCAT knockout were reversed; however, complementation was achieved by integration of multiple copies of the expression construct, resulting in marked overexpression of LCAT. We assessed replication and virulence in two independent knockouts, including one created in the RH $\Delta ku80$ background, which allows more precise disruption and complementation. We can not rule out that replication and virulence phenotypes in the new $\Delta lcat$ strains were blunted by compensatory changes in expression of other genes that dictate egress. However, a recent genome-wide mutagenesis screen revealed that parasites lacking

LCAT were not at a competitive disadvantage in culture ⁶⁰, supporting the conclusion that LCAT does not contribute substantially to parasite replication.

Work describing PbPL has shown an interesting observation of differential localization: sporozoites express PL on the surface, while in developing merozoites PL localizes to the PVM ^{34, 52}. This differential localization is consistent with the role of PbPL in sporozoite disruption of the HPM during cell traversal and merozoite rupture of the PVM during egress from hepatocytes. Work from the Coppens lab has shown a similarly interesting dual localization: soluble within the PV during intracellular replication, and shifting to the parasite surface following egress ³⁵. We attempted to visualize this apparent relocation of LCAT, but were unable to do so because: (1) anti-HA antibodies failed to detect LCAT-HA by immunofluorescence staining in our hands and subsequently by an independent group ⁹²; (2) our antibodies made to recombinant LCAT also did not work for immunofluorescence; and (3) LCAT endogenously tagged with a C-terminal YFP fusion showed an egress defect, suggesting that placement of a large tag at this location compromised LCAT function. Nevertheless, if LCAT redistribution occurs, several different scenarios can be envisioned for how LCAT contributes to calcium signaling and egress.

In one scenario, LCAT phospholipase activity principally functions by disrupting host-derived membranes, namely the PVM and HPM, before associating with the parasite surface. LCAT disruption of the PVM and HPM could promote influx of calcium from the medium to help potentiate calcium signaling in the parasite. Indeed, calcium influx into *Toxoplasma* tachyzoites was recently shown to occur via an L-type calcium channel, resulting in enhanced calcium signaling, microneme secretion and gliding

motility ²¹. LCAT disruption of the PVM and HPM would also allow influx of serum components including serum albumin, which enhances parasite calcium signaling, microneme secretion, and gliding motility via activation of PKG ⁶¹. Supporting this role, we observed a trend toward Δcat parasites maintaining an intact PVM for a longer time period than wild type parasites relative to peak calcium signaling. Although additional data collection is needed to authenticate the trend, the findings tentatively suggest that LCAT is either directly able to rupture the PVM, or facilitate the activity of other factors to attack this membrane.

In another scenario, LCAT could function on the parasite surface by generating products that enhance calcium signaling. LCAT phospholipase A2 activity generates lysophospholipids and fatty acids such as arachidonic acid (AA). In mammalian cells AA activates plasma membrane AA-regulated Ca^{2+} (ARC) entry channels, encoded by Orai2 and Orai3 proteins ⁶². Downstream metabolites of AA also activate certain plasma membrane transient potential channels including TRPV4 ⁶³. However, ARC and TRP channels are distinct from the aforementioned L-type calcium channel implicated in potentiation of calcium signaling in *Toxoplasma* via entry of environmental calcium. Additional studies are needed to distinguish the site of LCAT action and its precise role in potentiation of calcium signaling. Although our data do not definitively show that LCAT plays a role in calcium signaling due to variation between experiments, nevertheless there is a consistent trend showing a delay in calcium signaling in the strains lacking LCAT. One possible explanation for this is that between these experiments we purchased a different batch of zaprinast. The calcium signaling

phenotype was admittedly of modest strength, and the differences in zaprinast purity, concentration, etc. between the two batches may account for the altered results.

Along these lines, LCAT is closely related to lysosomal-phospholipase A2 (LPLA2), and the canonical LPLA2 lipase motif, AxSxG, is conserved. LPLA2 enzymes have been shown to become active at low pH ⁴⁰, and it has been reported the PV acidifies prior to egress ³⁹. Alternatively, the clue to regulation may lie in the proteolysis LCAT undergoes. Recently several members of the dense granule secretagogue have proven to be processed at PEXEL motifs in an aspartyl protease dependent manner. While it is true that many of these proteins contain the PEXEL signal in their N-terminal regions, LCAT does contain a PEXEL motif RRLEE starting at amino acid 594, and cleavage at this location would create proteolytic products of approximately the size seen in this study (**Figure 2-1D**). Cleavage of PEXEL containing motifs has been purported to drive localization to the PVM ⁶⁴.

Finally, it remains to be seen if a cooperative function exists between LCAT and other egress effectors, such as PLP1 and GRA41. As described by Hybiske and Stephens ³⁶, many intracellular pathogens co-opt pore-forming toxins (PFT) and phospholipases to escape membrane entrapment. Also worth noting are the parallels we see in *Plasmodium spp.*, with stage specific expression of different PFTs and stage specific activity of PbPL (sporozoite transmigration vs. merozoite egress). While in these systems a direct role has not been established for cooperativity between these two molecules, there are established examples of PFTs directly enhancing phospholipase activity, as is the case in bee venom ⁶⁵. As for GRA41, a recent study showed that a mutation in this dense granule protein results in aberrations of parasite

calcium levels and egress. Parasites lacking GRA41 exhibit dysregulation of calcium ion uptake, leading to altered calcium ion homeostasis and premature egress⁶⁶. Although LCAT and GRA41 appear to affect egress in opposite ways, they both reside in the PV, suggesting an emerging role for PV resident proteins in *Toxoplasma* egress.

2.4 Materials and Methods

Ethics statement

This study was carried out in strict accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and Association for the Assessment and Accreditation of Laboratory Animal Care guidelines. The animal protocol was approved by the University of Michigan's Committee on the Use and Care of Animals (Animal Welfare Assurance #A3114-01, protocol #09482). All efforts were made to minimize pain and suffering of the mice.

Parasite culture

Toxoplasma tachyzoites were maintained by serial passaging and growth in human foreskin fibroblasts (HFF) cells. Cell cultures were grown in Dulbecco's Modified Eagle Media (DMEM, source) supplemented with 10% fetal bovine serum (FBS, source), 2mM glutamine and 10 mM HEPES and grown in 5% CO₂ at 37°C. Parasites were liberated by scraping the infected HFF monolayer and passage through a 27G needle. The liberated parasites were then filtered through a 3 µm size filter (Millipore), counted on a hemocytometer, and added to HFFs at the appropriate density. For routine culture of

the parasites, 5 drops of naturally egressed parasites were passed into fresh host cells in a T25 flask.

Creation of transgenic strains

The knockout construct was constructed via fusion PCR. 5' and 3' homologous flanks were amplified from RH gDNA using ajsP1 + ajsP22 and ajsP6 + ajsP23, respectively. The *dhfr* selectable marker was amplified from pYFP.LIC.DHFR (Addgene) with ajsP20 + ajsP21. These three products were used as template and fused via a final PCR reaction with ajsP2 and ajsP5. The linear vector was transfected into RH Δ ku80 parasites and stable clones were isolated based on pyrimethamine resistance. The complement construct (pLCAT.Ku80.HXG) was created via Gibson cloning (In-Fusion, Clontech). The vector pM2AP.Ku80.HXG was prepared by double digestion with *Ascl* + *SpeI* and purified by gel extraction (Qiagen). The 5' and 3' *lcat* flanking sequences were amplified from RH gDNA with primer pairs ajsP62 + ajsP52, and ajsP63 + ajsP59, respectively. The *lcat* cDNA was amplified from a cDNA library with primer pair ajsP53 + ajsP58. The catalytically dead construct, pLCATs332a.Ku80.HXG, was generated using site-directed mutagenesis (QuikChange XL, Agilent Technologies) and primers ajsP64 + ajsP65. The constructs were linearized prior to transfection by double digest with *KpnI* + *ApaI* and transfected into RH Δ ku80 Δ *lcat* parasites, and stable clones were isolated based on resistance to MPA + Xan. For creation of RH Δ *lcat* lines using CRISPR-Cas9, 20bp of *lcat* specific guide sequence was inserted into pCRISPR-Cas9-Ble, using site-directed mutagenesis and the primer pair ajsP200 + ajsP201. PCR was used for genetic confirmation shown in **Figure 2-1** as follows: amplification across exon1 & exon 2 (A)—

ajsP15 + ajsP138; integration of the 5' end of the complement construct at the *ku80* locus (B)—XhoI_Ku80_5'Flank.f + ajsP138; integration of the 3' end of the complement construct at the *ku80* locus (C)—ajsP19 + XhoI_Ku80_5'Flank.r.

Plaque assay

Infected monolayers in a T25 were washed 3X with 37°C PBS prior to scraping and host lysis as described above. Parasite suspensions were made via serial 10-fold dilution to reach a concentration between 75 – 150 parasite / 150 µl PBS. Monolayers of HFFs in individual 6-well plates were immediately inoculated with 150 µl of the parasite solution. The infected monolayers were then incubated, undisturbed, for 7 days prior to fixation with 2% crystal violet. Plaques were quantified using ImageJ on wells that had been digitally scanned with an included ruler for setting the scale.

Replication assay

Infected HFFs were fixed with 4% paraformaldehyde at either 16 or 32 hours post infection. The fixed monolayers were permeabilized with 0.01% Triton X-100 and labeled with rabbit anti-SAG1 and counterstained with DAPI. At least 250 vacuoles were quantified per strain per experiment.

Generation of LCAT specific polyclonal antibodies

The bacterial expression construct pET15b was linearized with inverse PCR (iPCR) with primer pair ajsP142 + ajsP144. The *lc* coding sequence was amplified with ajsP143 +

ajsP145 from a *Toxoplasma* cDNA library, and was subcloned into pET15b via Gibson assembly.

Virulence assay

Groups of 5 female BALB/c or Swiss Webster (Jackson) female mice, aged 6 weeks, were infected with either 10 or 50 *T. gondii* tachyzoites subcutaneously in 150µl of PBS. Delivery of an accurate dose of infectious parasites was confirmed by performing plaque assays in parallel (described above). In the event of mice surviving the infection, seropositivity was tested by enzyme-linked immunosorbent assay (ELISA).

In vitro competition assay

RHΔ/*cat* parasites were transfected with a GPI-anchored GFP variant and stable clones were selected following treatment with pyrimethamine. Extracellular parasites were counted on a hemocytometer and roughly equal numbers (5×10^5) of both RH and RHΔ/*cat*GFP parasites were co-inoculated into a T25 of HFFs. Following lysis of the host cell monolayer extracellular parasite were collected, filtered, and fixed on poly-L lysine coated glass slides. Parasites were immunolabeled with rabbit anti SAG1, and enumerated via fluorescence microscopy. This process was repeated for a total of 3 passages.

Egress assay (LDH)

30 h prior to assay, infected monolayers in a T25 were washed 3X with 37°C PBS prior to scraping and host lysis as described above. Following centrifugation, parasites were

resuspended to a density of $5 \times 10^5/\text{ml}$ and 100 μl were used per well (5×10^4 tachyzoites). Prior to egress assays, infected wells were washed 3X with 37°C Ringer's buffer, after the final wash cells were treated with either 57 μM zaprinast diluted in Ringer's, or Ringer's solution with an equal volume of DMSO. The treated plates were allowed to incubate at 5% CO_2 and 37°C for 20' before removal and immediate placement on ice. 50 μl of the supernatant was removed and placed into individual wells of a 96-well round bottom plate. The round bottom plates were centrifuged at 4°C at 500g, for 5 minutes. 30 μl of solution was removed and stored at 4°C, and used within 1 hour as source assay material for LDH content (BioVision).

Calcium signaling

All calcium signaling experiments utilized parasites expressing cytosolic GCaMP (courtesy of Silvia Moreno, University of Georgia). 30 h prior to assay, infected monolayers in a T25 were washed 3X with 37°C PBS prior to scraping and host lysis as described above. 5×10^4 tachyzoites were added to confluent monolayers in an 8-well chamber slide (Ibidi), and allowed to replicate. To observe calcium signaling during induced egress, infected monolayers were washed 3X with 37°C Ringer's buffer, after the final wash 100 μl of Ringer's buffer was added to each well. Imaging plates were placed in an environmental chamber heated to 37°C on a Zeiss Axio inverted microscope equipped with an AxioCam MRM CCD camera using a 20X objective. Zaprinast was added at a 200 μM final concentration by adding 100 μl of a 400 μM solution in Ringer's buffer. Acquisition was captured at a frame rate of 2-3s for 4 minutes total.

mCherry Release

For assays examining the integrity of the PVM, 5×10^7 purified RH and RH Δ /*cat* parasites expressing cytosolic GCaMP were transfected with 50 μ g of a plasmid expressing two copies of mCherry under the tubulin promoter (pTub mCherry_mCherry). Following transfection, 1×10^6 parasites were added to confluent HFF monolayers in glass bottom chamber slides (Ibidi), and allowed to replicate for 30 hours. Egress induction and image acquisition were identical to the calcium signaling assays previously described, with the following exceptions: 1) 2 μ M cytochalasin D was included in the induction media to paralyze egressing tachyzoites, and 2) in addition to using a GFP filter cube, images were also collected using a Texas Red cube to observe the mCherry signal in the PV.

Chapter 3

LCAT processing and activity

3.1 Introduction

Within the Apicomplexan parasites, *Plasmodium falciparum* is the most infamous due to massive negative impact on human health—over 2 billion people are exposed to this parasite every year ⁶⁷. One reason underlying these parasite's ability to accomplish such a massive feat, is their capacity to interact with and modulate the infected host cell; a process mediated by secreted, exported proteins ⁶⁸. In order for this process to occur, the parasite proteolytically processes secreted proteins to drive their interaction with the host cell ⁶⁹. In *Plasmodium spp.* the details of this process that are best understood involve three aspects: 1) a *Plasmodium* export element (PEXEL), which is a pentapeptide motif of RxLxE/Q/D (x is any amino acid) that serves as a cleavage motif ^{68, 70}; 2) an aspartyl protease, Plasmepsin V that serves as a maturase by recognizing and cleaving PEXEL motifs ⁷¹; and 3) a translocon that exports cleaved PEXEL-containing proteins across the PVM into the host cytosol ^{72, 73}. Despite the close relationship between *Toxoplasma* and *Plasmodium spp.* a parallel process in *T. gondii* has remained elusive until recently.

Following host cell invasion by the *Toxoplasma* tachyzoites, the parasite resides within a parasitophorous vacuole (PV) that does not fuse with host cell membranes, including host lysosomes ⁷⁴. Unlike protein secretion from the micronemes or the rhoptries, proteins from the dense granules (GRA proteins) are secreted post invasion while the parasite resides within the PV. While the functions of GRA proteins are quite diverse, they have traditionally been implicated in the establishment of the vacuole as a replicative niche ⁷⁵. With the recent discovery of GRA protein processing, a novel role for dense granule proteins has been uncovered in host:parasite interactions. GRA15, 16, and 24 have recently been reported to be processed and exported into the host cell where they alter host gene expression ⁷⁶⁻⁷⁸. Additionally, GRA19, 20, and 21 are processed but instead of export into the host cell they are subsequently targeted to the parasitophorous vacuolar membrane (PVM) ⁶⁴. Furthermore, the processing and export of these GRA proteins primarily occurs through machinery that is homologous to those initially observed in *Plasmodium spp.* ^{64, 79-81}. There is at least one alternative pathway for protein export and processing ⁷⁸, but so far the significance between the two has not been uncovered.

Along these lines, *Tg*LCAT is among the subset of dense granule proteins that are proteolytically processed ³⁵. The nature of this event remains uncharacterized, however the predicted cleavage site based off the size of the processed fragments, is located near a PEXEL motif and falls within a unique domain. LCAT contains an approximately 140 amino acid inserted element (465 – 605) within the conserved catalytic PLA₂ domain, this insert is not conserved in other members of the Apicomplexa

phylum—and is only seen within the coccidian subgroup as all other homologues encode intact domains.

TgLCAT is predicted to be a member of the PLA2 family, which consist of a group of enzymes that are unrelated in their structure and vary in their mechanisms of lipid hydrolysis, but share the common function of sn-2 hydrolysis of the central carbon of phospholipids⁹³. This group is diverse and contains secreted PLA2s, cytosolic PLA2, and lysosomal phospholipase A2, among others. One of the common features shared among these molecules and *TgLCAT* is the presence of an alpha/beta hydrolase fold and a Ser/His/Asp catalytic triad. The alpha/beta hydrolase fold is characterized by a central beta sheet of 7 parallel strands interspersed with alpha helices on both sides⁹⁴. The catalytic mechanism of these enzymes is similar to that of trypsin, despite the lack of similarity in sequence and function between the two. Substrate binding positions the oxygen of the carbonyl group in the oxyanion hole, then a charge-relay system through the catalytic triad activates the oxygen atom of the catalytic serine that is responsible for nucleophilic attack. The histidine residue then protonates the leaving group, with the H⁺ ion from nucleophilic attack⁹⁵.

Outside of the conserved alpha/beta hydrolase fold, *TgLCAT* shares another common feature of PLA2 enzymes, the cap domain. The cap domain is found above the catalytic site, and blocks access in the absence of a substrate. One common feature of all lipases is their activity at the lipid/water interface, and in PLA2 enzymes with cap domains, binding to a target membrane generally induces a conformational change that allows substrate access by removal of the cap blockage⁹⁶. Little is known specifically about the activation of LPLA2 enzymes, but many lipases are activated by a

phenomenon known as “interfacial activation” which occurs when an enzymes binds to a lipid membrane—and many lipases are more active when substrates are presented in the form of liposomes or micelles ⁹⁷. One thought for how this may occur is that in enzymes that possess a mobile “lid” element, binding to a lipid interface displaces the lid element and exposes hydrophobic elements of the enzyme that further drive association of the protein with target membranes ^{98,99}.

In this study we attempt to explore the aspects involved in *Tg*LCAT processing and the biochemical nature underlying them.

3.2 Results

3.2.1 LCAT structure and phylogeny

The LCAT ORF is located on chromosome VII of *T. gondii*, and codes for a protein of 763 amino acids in length. The protein has a conserved phospholipase A2 (PLA₂) domain, and this domain shares a high degree of homology for two types of PLA₂: lysosomal phospholipase A2 and lecithin cholesterol acyltransferase (34% vs. 31% respective homology with *H. sapiens*). *Tg*LCAT contains a conserved pentapeptide motif, AHSLG, that is characteristic of serine lipases (**Figure 3-1A**). The serine residue of this motif forms the acylated center of the catalytic triad (SDH; serine/aspartate/histidine), and these residues are found across the phylum. *Tg*LCAT is well conserved within the Apicomplexan's, with the coccidian members of the phylum forming a subgroup (**Figure 3-1B**). This sub group is likely due to the presence of an inserted domain at positions 465-605, which is only seen among the coccidians. This insert effectively splits the conserved PLA₂ domain of *Tg*LCAT into two entities. In addition to these features, *Tg*LCAT also contains a signal peptide that targets the

protein to the parasite's secretory pathway, and an N-terminal domain that shares no homology with known proteins (**Figure 3-1C**).

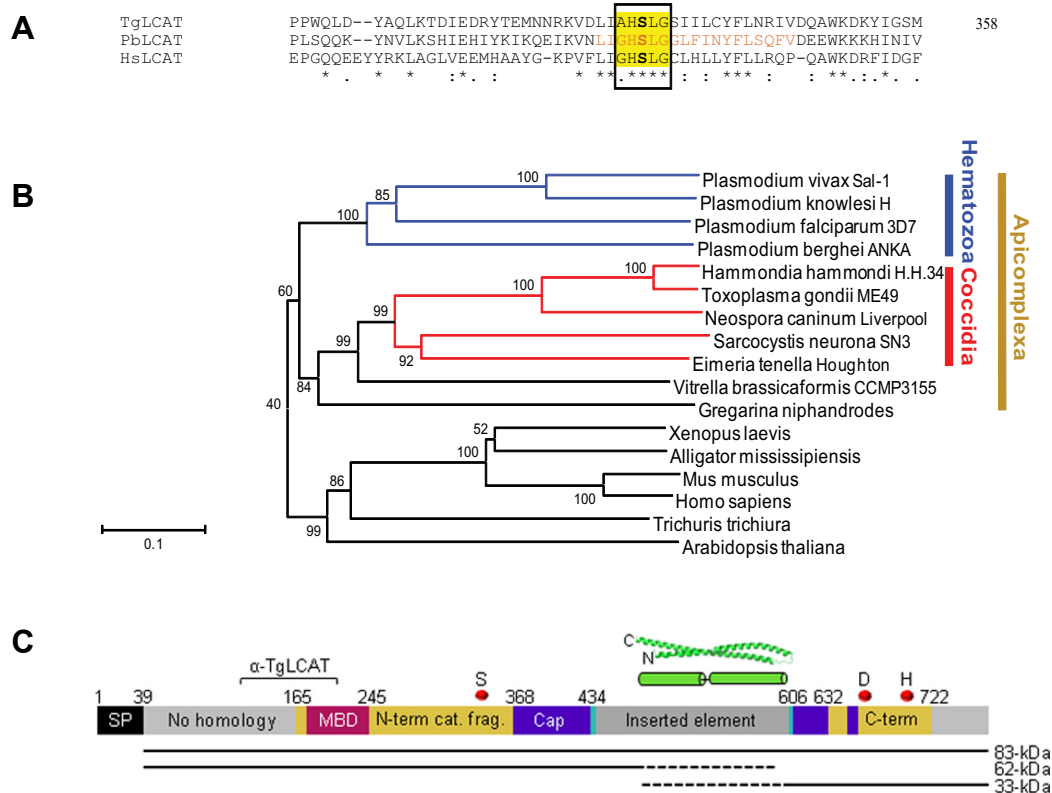


Figure 3-1. Phylogeny and schematic of TgLCAT.

(A) Multiple sequence alignment of the conserved lipase domain of TgLCAT homologues, species shown: *Toxoplasma gondii*, *Plasmodium berghei*, *Homo sapiens*. (B) sequence relationships among LCAT family proteins. Unrooted phylogenetic tree of the LCAT family proteins constructed using neighbor joining analysis. Numbers at the branch nodes represent bootstrap values (as percentages) obtained in 1,000 replications. Branch lengths indicate the number of amino acid differences. (C) schematic illustration of TgLCAT. Predicted domain structure of TgLCAT is shown with putative signal peptide (SP), membrane-binding domain (MBD) based on homology with human LPLA₂, N-terminal catalytic fragment (N-term cat. frag.), cap domain (Cap), inserted element (IE), and C-terminal catalytic fragment (C-term). Numbers above the illustration indicate amino acid position at the beginning of select domains. Position of the recombinant polypeptide used to immunize rats for anti-TgLCAT (-TgLCAT) is indicated. Positions of catalytic residues SDH are indicated with red spheres. A putative coiled-coil domain within the inserted element is shown based on the highest scoring *ab initio* model from I-TASSER. The precursor TgLCAT species and putative proteolytic fragments are indicated as black lines below the schematic, with dashed lines indicating the estimated region of proteolysis.

3.2.2 LCAT is processed

LCAT is expressed by the tachyzoites and is readily detected by SDS-PAGE and western blotting; surprisingly, LCAT is not detected as a whole fragment but is instead

seen as two distinct species: a full-length and a processed form—the result of proteolytic cleavage of the protein (**Figure 2-1**). Immunoblots of parasite lysates reveal a full-length band of ~105 kDa, and a smaller band ~38 kDa which represents the processed C-terminal fragment. Interestingly the size of the observed full length product differs from the predicted size (~83 kDa).

We next sought to determine if the processing of LCAT would destroy the enzyme's ability to function, and to aid in our understanding and visualization of this aspect we attempted to model the tertiary structure of *Tg*LCAT. The modeling was done using the recently solved *Homo sapiens* LPLA2 structure as a template⁹¹, which was a higher scoring computational template for threading than *Hs*LCAT. The predicted model of *Tg*LCAT shows the formation of an active site with residues that would remain in close proximity to one another based on folding of the core catalytic domain, and the presence of disulfide bridges spanning both fragments of the protein that would effectively pin the enzyme together following cleavage (**Figure 3-2**). The location of the inserted element maps to a conserved cap region, which based on predictions would be unlikely to interfere with substrate access. *Tg*LCAT contains two predicted PEXEL motifs RDLLQ at position 402 and RRLEE at position 587, and cleavage near position 587 would roughly account for the size of the processed fragments. Additionally, when intracellular parasites were treated with DSP, a cell-permeant cross linker, LCAT migrated as a single species when detected by western blot³⁵, indicating that even after cleavage the two LCAT fragments remain in close proximity, likely as a non-covalent complex.

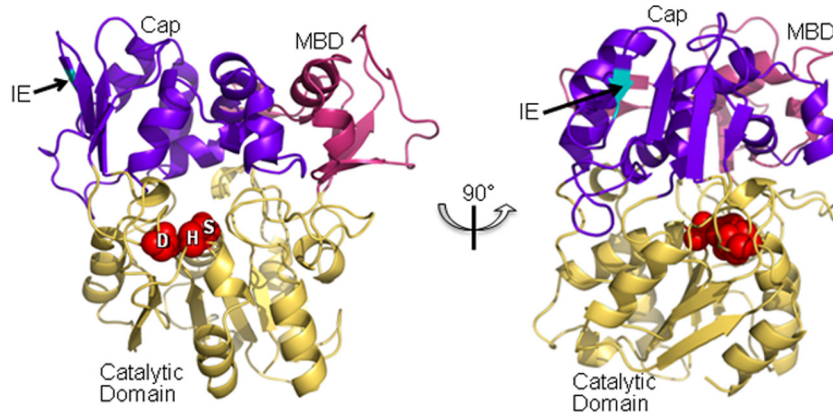


Figure 3-2. Structural model of *TgLCAT*. Model was constructed with Phyre2, which identified human LPLA₂ (Protein Data Bank code 4X91) as the highest scoring template for threading. Domains are colored according to the published structure of human LPLA₂ (34). The position of the inserted element is indicated with an *arrow*, with the two flanking amino acid backbone residues shown in *cyan*. Catalytic residues, including their side changes, are shown as *spheres* and labeled with the single letter designations for the corresponding amino acids. The image on the *right* is viewed from the perspective of the membrane, with the catalytic pocket shown centrally. The *left image* is rotated 90° from the *right image*.

3.2.3 LCAT is not processed in extracellular parasites

In order to gain more insight into the processing event and to further characterize the role that processing plays in the secretion and activity of *TgLCAT*, we generated mouse polyclonal antibodies against the secreted form of the protein (residues 39-763). Thirteen BALB/c mice were immunized and thereafter boosted with recombinant *TgLCAT*, and were then tested by ELISA for LCAT affinity (**Figure 3-3A**). The sera showing the highest affinity for the recombinant protein were pooled and then tested for specificity via western blotting on wild-type and Δcat parasite lysates. Initial blots with the polyclonal antibody showed a high degree of non-specific background staining, which was significantly reduced following affinity purification of the pooled sera (**Figure 3-3B**). Unfortunately, we were still only able to detect the full-length and the C-terminal

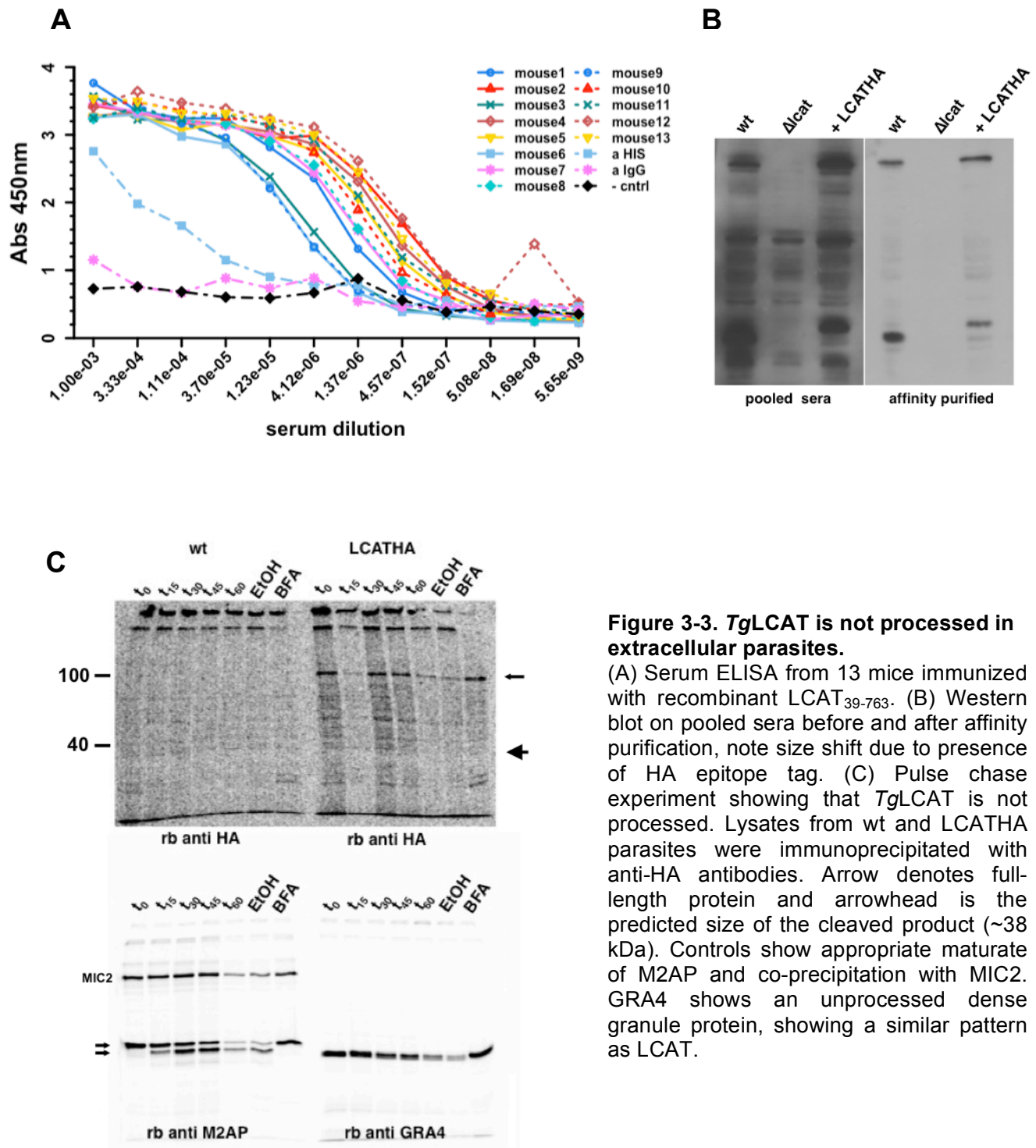


Figure 3-3. *Tg*LCAT is not processed in extracellular parasites.

(A) Serum ELISA from 13 mice immunized with recombinant LCAT₃₉₋₇₆₃. (B) Western blot on pooled sera before and after affinity purification, note size shift due to presence of HA epitope tag. (C) Pulse chase experiment showing that *Tg*LCAT is not processed. Lysates from wt and LCATHA parasites were immunoprecipitated with anti-HA antibodies. Arrow denotes full-length protein and arrowhead is the predicted size of the cleaved product (~38 kDa). Controls show appropriate maturation of M2AP and co-precipitation with MIC2. GRA4 shows an unprocessed dense granule protein, showing a similar pattern as LCAT.

product via western blot, as the larger N-terminal fragment remained elusive. Furthermore, this antibody proved to be insufficient for immuno-staining, as indirect immunofluorescence assays against the wild-type and Δcat infected monolayers showed no specific staining.

Next we sought to determine the spatial and temporal aspects that govern *Tg*LCAT processing by examining this process in close detail using radio-labeling and pulse chase experiments. After a 15 minute metabolic labeling with [³⁵S]methionine/cysteine, samples were collected every 15 minutes, followed by LCAT immunoprecipitation using HA antibodies. Surprisingly, these experiments revealed that LCAT is not processed while in intracellular parasites in the first 60 minutes following protein synthesis (**Figure 3-3C**).

3.2.4 LCAT transacylase activity

Our final attempts to characterize *Tg*LCAT involved expression of the protein recombinantly for biochemical analysis. We chose to clone the secreted form of the protein (39-763) into a baculovirus expression vector suitable for driving expression from insect cells, based off previous reports for this protein. This approach generated an extremely limited amount of recombinant protein that displayed some activity. Initial experiments assayed transfer of radiolabeled phospholipid to ceramide (**Figure 3-4A,B** LPLA2 activity), and were positive for activity with the wild-type version of *Tg*LCAT but not with a catalytically inert form of the enzyme (**Figure 3-4C,D**). However, due to limitations from the low amount of protein and issues with protein stability, these results could not be followed up on or reproduced.

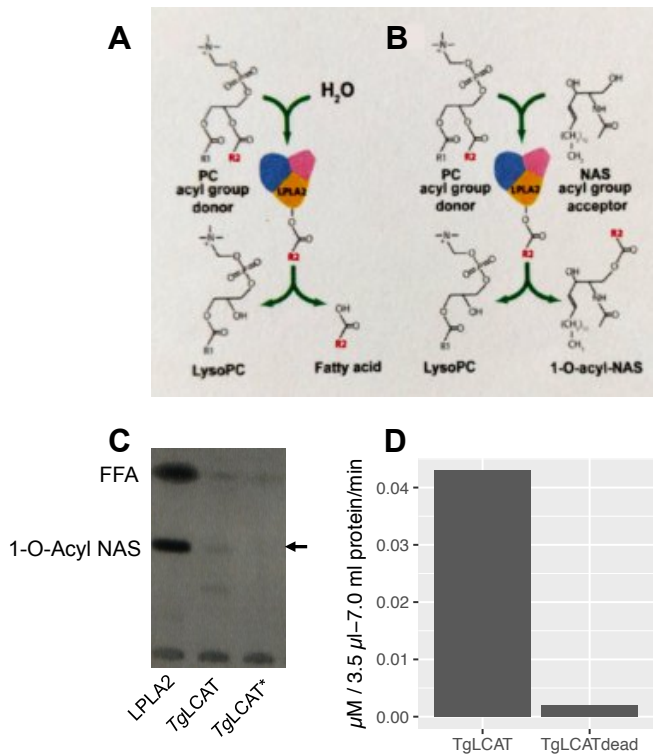


Figure 3-4. LCAT transacylation activity.

(A) Schematic illustrating the hydrolytic activity of LPLA2 in the absence of an acceptor molecule. (B) Schematic illustration the transacylation reaction of LPLA2 in the presence of the acceptor ceramide molecule. (C) Gel showing the transacylation driven by *Tg*LCAT. DOPC/sulfatide/NAS liposomes (10/3/1 molar ratio) were used and incubated with cell homogenate (LPLA2) or purified protein for 60 minutes at 37°C. The HPTLC plates were developed in a solvent of chloroform:acetic acid (90/10 v/v). Ceramide (18-Avanti) standards were used to calculate LPLA2 activity. LPLA2 = human cell lysate; *Tg*LCAT = wild type ; *Tg*LCAT^{dead} = catalytically inactive mutant. FFA = free fatty acid; 1-O-Acyl NAS = acylated product.

3.3 Discussion

Microbial PLA₂ enzymes have long been recognized for their contributions to pathogenesis and cytotoxicity. Here we have examined a recently discovered parasitic PLA₂ that contains novel characteristics regarding its structure and maturation. *TgLCAT* is highly similar to both LPLA2 and LCAT enzymes, and more work is needed to definitively characterize the activity of this molecule. The conserved lipase domain, AHSLG present in *TgLCAT* is more predictive of LPLA2 enzymes than of LCAT enzymes⁸², and we have generated limited data that supports this case as we have observed catalytically dependent transferase activity to ceramide. It is important to recognize however that this data was not thoroughly replicated due to conditions that we were unable to circumvent. Existing published data shows that *TgLCAT* has transferase activity to cholesterol³⁵; however in the original study transferase activity specific to ceramide was not tested. To date there have been no descriptions of PLA₂ enzymes that exhibit a dual transferase activity to both ceramide and cholesterol, but given the existing data this enzyme may be capable of catalyzing both reactions. *TgLCAT* is unique in that it belongs to a subclass of PLA₂ enzymes that are oddly split by the insertion of a 140 amino acid insert that interrupts the conserved phospholipase domain. This insert is only conserved among the coccidians, and may hold the clues necessary for determining key differences between the homologues in terms of their localization (surface vs. secreted) and the processing observed in *Toxoplasma*.

The processing itself is an interesting event, as recent data from several different groups have uncovered a pathway for dense granule processing that involves a PEXEL

cleavage signal,⁶⁴ and a maturase, ASP5⁷⁸⁻⁸⁰. Interestingly, *Tg*LCAT contains a PEXEL domain, RRLEE at position 587. Recent work has described PEXEL motifs starting with “RRL” residues as preferential signals for ASP5-mediated proteolysis⁷⁹, and consistent with this observation, LCAT has recently been reported to be processed by ASP5 at a non-canonical RRLxx motif at position 476. This was discovered in a proteomic screen identifying and sequencing the N-terminus of processed proteins⁹². The more interesting question regarding LCAT processing is what affect this has on activity. The only Apicomplexan homolog to *Tg*LCAT that has been studied is the *Plasmodium berghei* *Pb*PL. Outside of the unique inserted element, these two homologs are closely conserved, yet *Pb*PL is active without a cleavage event³⁴, and *Tg*LCAT is apparently active while it is cleaved or uncleaved³⁵. Cleavage of the enzyme may simply target the protein to the PVM/IVN as previously proposed⁶⁴. The inserted element lacks homology to any known proteins; however, *in situ* modeling of this peptide using iTasser software (University of Michigan) predicts the formation of a coiled coil domain. As the RRLx PEXEL motif lies just at the edge of this domain (insert: 465-605; RRLxx: 467), cleavage at this location may release the coiled-coil and provide steric freedom to drive protein:protein interactions or correctly position the enzyme⁸³.

Alternatively, we need to consider that there exists a possibility that *Tg*LCAT is not cleaved at all, or is only cleaved while the parasites are intracellular. Following metabolically labeled protein over the course of 1 hour, we were not able to detect cleavage in extracellular parasites. While these results are apparently at odds with what has been recently reported⁹², it is possible that ASP5 is preferentially active while the parasites are intracellular—and thus explain why we were unable to observe processing

during pulse-chase experiments but were consistently able to observe processing in western blots, where the lysates are made from intracellular parasites. On the other hand, we also see variable levels of processing from western blots, which suggest that proteolysis is happening during sample preparation. One possible explanation for this is that another secreted protease is responsible for the maturation of *Tg*LCAT, and these two partners are being artificially exposed during lysate preparation. If this were the case, a secreted protein from the micronemes could rapidly convert a PV with accumulated full-length *Tg*LCAT into its processed form just prior to egress. There are indeed proteases secreted from the micronemes (TLN4, SUB1)^{84, 85}, which could serve this purpose. Regardless, more detailed characterization on the role of processing in relationship to LCAT activity is required.

3.4 Materials and Methods

LCAT modeling and sequence analysis

Phyre2 was used for template-dependent modeling of *Tg*LCAT queried with amino acids 39–763 [minus the “inserted element” at 465-605], allowing the program to identify the best candidate template for modeling. Human lysosomal phospholipase A₂ (LPLA₂; Protein Data Bank code [4X91](#)) was the highest scoring template upon which the structural model was built. Models visualization was performed with PyMOL. For the phylogenetic analysis, LCAT orthologues were identified via BLAST search using the TGME49_272420 sequence from the EuPathDB. Representative eukaryotic sequences were chosen from major groups via BLAST of the non-redundant protein database (blast.ncbi.nlm.nih.gov). To assess the phylogenetic relationship between the

homologues, a multiple sequence alignment of LCAT proteins was performed using MUSCLE, from which a bootstrap neighbor-joining phylogenetic tree using p-distance method and 1,000 bootstrap support were generated (MEGA version 6.0 software). A schematic tree was predicted by neighbor-joining using the LCAT sequences from *Hammondia hammondi* (HHA_272420), *Neospora caninum* (NCLIV_034880), *Sarcocystis neurona* (SN3_00202440), *Eimeria tenella* (ETH_00005915), *Vitrella brassicaformis* (Vbra_21178), *Gregarina niphandrodes* (GNI_089950), *Plasmodium berghei* (PBANKA_112810), *Plasmodium falciparum* (PF3D7_0629300), *Plasmodium knowlesi* (PKH_111960), *Plasmodium vivax* (PVX_114565), *Trichuris trichiura* (GI:669226237), *Arabidopsis thaliana* (GI:30689946), *Xenopus laevis* (GI:148229443), *Alligator mississippiensis* (GI:944443680), *Homo sapiens* (AAB34898.1), and *Mus musculus* (AAL11035). R software (package 'ape') was used to draw the dendograms.

Lysate preparation and immunoblotting

Lysed parasites were filter purified, chased and washed with 4°C PBS, and resuspended to a concentration of 10^7 tachyzoites / 5 µl in 100°C sample buffer + 2ME. For SDS-PAGE separation, 5×10^7 tachyzoites were loaded per lane on a 10% gel, following electrophoresis samples were transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with 10% nonfat milk, probed with mouse anti-LCAT 1:3500, rat anti-HA 1:1000, or rabbit anti-actin 1:200000 in 1.25% nonfat milk + PBS-Tween 20 0.2%, washed in PBS-T, and probed with horseradish peroxidase conjugated secondary antibodies at 1:1000. Blots were incubated with enhanced

chemiluminescent substrate (Super Signal West Pico, Pierce) and exposed to film or captured with a chemiluminescent imager (Syngene).

Metabolic labeling and immunoprecipitation.

Pulse-chase labeling was performed as follows. T25 tissue culture flasks of confluent HFFs were infected with 3.5×10^6 freshly lysed tachyzoites. The following day parasites were liberated by mechanical lysis by serial passage through a 20G needle, and then purified in cysteine and methionine free DMEM (Gibco) + 1% FBS (Gibco) at 1×10^8 parasites / ml. Metabolic labeling was done at 37 kBq/ml of ^{35}S [methionine + cysteine] for 10 minutes at 37°C. Following labeling, samples were chased with 5 mM [methionine + cysteine] in DMEM + 1% FBS and 100 μl samples were collected at 0, 15, 30, 45, and 60 min. Parasites collected at each time point were then spun down and lysed in RIPA buffer (50 mM Tris-HCl [pH 7.5], 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS, 150 mM NaCl, supplemented with DNase and RNase) plus protease inhibitors (Roche), followed by immunoprecipitation with rat anti-HA, rabbit anti-M2AP, or rabbit anti-GRA4 antibodies. Following immunoprecipitation, samples were run on 10% SDS-PAGE gels, which were then incubated in Amplify (Amersham) and dried in cellophane. Dried gels were exposed to a phosphoimaging screen overnight and imaged on a Typhoon Imaging system.

Baculovirus expression and cloning

The *Tg*LCAT coding sequence was amplified from *Toxoplasma* cDNA using the following primers ajsP113 TACTTCCAATCCAATGCATTGTCGTCGCCTTTCTCC, and

ajsP114 TTATCCACTTCCATTACGCGTAGTCCGGGAC. The resulting amplicon (2 kb) was gel purified and LIC cloned into baculovirus expression vector Ac7, provided by Clay Brown at the High Throughput Protein Lab at the University of Michigan, following the protocol described previously ⁸⁶. Following cloning, protein was expressed by the High Throughput Protein Lab in 10L batches, and affinity purified using the N-terminal HIS tag.

Ethics statement

This study was carried out in strict accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and Association for the Assessment and Accreditation of Laboratory Animal Care guidelines. The animal protocol was approved by the University of Michigan's Committee on the Use and Care of Animals (Animal Welfare Assurance #A3114-01, protocol #09482). All efforts were made to minimize pain and suffering of the mice.

Parasite culture

Toxoplasma tachyzoites were maintained by serial passaging and growth in human foreskin fibroblasts (HFF) cells. Cell cultures were grown in Dulbecco's Modified Eagle Media (DMEM, source) supplemented with 10% fetal bovine serum (FBS, source), 2mM glutamine and 10 mM HEPES and grown in 5% CO₂ at 37°C. Parasites were liberated by scraping the infected HFF monolayer and passage through a 27G needle. The liberated parasites were then filtered through a 3 µm size filter (Millipore), counted on a hemocytometer, and added to HFFs at the appropriate density. For routine culture of

the parasites, 5 drops of naturally egressed parasites were passed into fresh host cells in a T25 flask.

Transacylase activity assays

Recombinant protein was incubated with DOPC/sulfatide/NAS liposomes at 37°C at pH 4.5 by the Shayman lab as described previously⁸⁷.

Chapter 4

Discussion and Future Methods of Studying Egress

4.1 Discussion

Egress from the infected host cells represents one of the key events in the intracellular lifecycle of all intracellular pathogens. For *Toxoplasma gondii*, and its *Plasmodium spp.* relatives, this process is accomplished via a cytolytic mechanism. Despite the prominence of this step and the downstream consequences of host damage, little is known about this event mechanistically. The detailed molecular mechanisms of egress have been slow to emerge, and currently only one egress effector protein, PLP1, has been mechanistically characterized. This work focused on determining the role of another secreted egress effector, *Tg*LCAT, during this enigmatic process. While the mechanistic role of *Tg*LCAT has remained elusive, this work has led to an important clarification of the existing literature by showing that *Tg*LCAT does not have a role in virulence or parasite replication, but instead this protein primarily acts during egress—and its phenotype may be tied to a shift in the calcium signaling pathway.

Given the subtle phenotype seen during *in vitro* assays, and the lack of a phenotype altogether during *in vivo* experiments, further pursuit of a mechanistic role for

*Tg*LCAT during toxoplasmosis is difficult to justify. However, this molecule does contain an interesting feature that is worth further exploration: the unique inserted element that interrupts LCAT's PLA₂ domain. The presence of this element in a subpopulation of the apicomplexans suggests that in the coccidians, this element may be responsible for differences in activity between groups, i.e. transmigration in *Plasmodium spp.* vs. egress in *Toxoplasma*. Assuming the inserted element is responsible for regulating LCAT activity during intracellular replication, testing the consequence of deleting the element entirely (LCAT vs. LCAT Δ_{insert}), and the effect of adding this element to heterologous proteins of known function (characterized GRAs, fluorophores) could reveal important information in terms of change of protein function or localization. While these discoveries would not likely be of high impact in regards to *Tg*LCAT, these insights could lead to the development of valuable tools—for example by allowing researchers to design experiments that differentially regulate the activity or localization of a protein of interest, for example. Additionally, this impact of *Tg*LCAT may be confined to certain stages of the parasite. For example, the *Plasmodium* homologue of *Tg*LCAT seems to function differently in the sporozoites (transmigration)³⁴ than in the liver-stage (egress)⁵². It is therefore a possibility that *Tg*LCAT has a relatively minor role in the tachyzoites, and a more prominent role in the *Toxoplasma* bradyzoite or sporozoite—stages that are currently very difficult to work with in the laboratory. It is puzzling that to date only PLP1 has been identified as a “true” egress effector. Despite the work of several labs conducting independent forward genetic screens to identify egress factors, these studies have reliably uncovered proteins that are involved in calcium signaling and/or microneme secretion. The simplest explanation for this

observation is that only PLP1 and parasite motility (motility is a result of microneme secretion) are needed for tachyzoite egress in *Toxoplasma*. One could argue that egress is still a vital process in the lifecycle based on the profound loss of virulence in the PLP1 knockout strains. Mice that are infected with $\Delta plp1$ tachyzoites, when compared to wild-type, exhibit a $\geq 10^5$ fold loss in virulence¹⁰. However, it is important to note in this study, there were similar parasite loads between mice infected with wild type vs. $\Delta plp1$ parasites, which indicates that despite the egress phenotype seen during *in vitro* assays, $\Delta plp1$ mutants are clearly able to effectively complete the lytic cycle *in vivo*. Furthermore, during routine tissue culture passage of these lines, there are no defects in the ability of $\Delta plp1$ parasites to complete the lytic cycle as measured by parasite outgrowth. While it is true that $\Delta plp1$ parasites form smaller plaques when grown on a cell monolayer, these results also have an alternative explanation; parasites that struggle to egress ($\Delta plp1$), have a secondary invasion defect¹¹. Meaning that when $\Delta plp1$ parasites are allowed to naturally egress (vs. when they are mechanically liberated from a monolayer), there is a defect in the ability of the naturally egressed parasites to re-invade a host cell. Therefore, the defect in plaque size seen in the $\Delta plp1$ parasites cannot be specifically attributed to defects in egress or defects in invasion. It is possible the smaller plaques are a result of an invasion defect, because it is not apparent how a failure to egress from a monolayer of fibroblasts would be consequential—as these cells lack the innate defense mechanisms to clear *T. gondii*. Important questions to consider include: what is the fate of parasites that are unable to egress? Infected cells typically undergo a necrotic cell death—in which case the parasite would become extracellular. In other cases, parasites that fail to egress could

presumably be targeted by phagocytic cells—and even the result of this is not directly clear, some strains of *T. gondii* have demonstrated an ability to subvert phagocytosis. This process allows the parasites to “invade” from within the phagosome and form a novel, productive PV inside which the parasite can replicate. Therefore even in the event of engulfment by phagocytes, *T. gondii* can still form a novel PV and turn the tables by parasitizing these immune cells.

T. gondii has an actinomycin motility system that has long been recognized for its contributions to both invasion and egress. However, work done using chemically paralyzed parasites revealed that in the absence of motility, the tachyzoites were still able to egress by lysing the host cell. While these results remain compelling, it is important to recognize that in these experiments it would be very difficult to distinguish parasite egress from host cell necrosis. For example, in these experiments, infected monolayers were treated for a prolonged period (10 minutes) with A23187, a calcium ionophore ¹⁷. It is difficult to determine if the egress observed in these cases were due to parasitic secretion of effector proteins, or if the chemical treatment lead to host cell death, or even a combination of the two. Reportedly, A23187 is highly toxic to mammalian cells when treated at slightly higher doses (10-fold) for 60 minutes, and this toxicity is presumably a result of plasma membrane damage ⁸⁸. It is important to recognize recent work and how these data fit into the current egress model as well. Recent work by the Meisner group has involved making gene knockout for components of the motor system in *Toxoplasma*, including *actin* and *myosin*. These genetically paralyzed tachyzoites are still able to invade but are unanimously unable to egress ¹⁸.

Regardless of whether or not egress is a crucial process to the tachyzoite lifecycle, or a dispensable one, it is plausible that studying egress *in vitro* via the commonly used assays in our field is not ideal. There are at least three shortcomings regarding the current *in vitro* models for egress. First, the cell line most commonly used for both routine culture and most *in vitro* experiments is the human foreskin fibroblast (HFF). This is done primarily out of convenience—these cell lines are easy to maintain in culture and are ideal for microscopy because of their flat morphology. However, this is not a cell type that *Toxoplasma* will likely infect in the course of a natural infection. Although *Toxoplasma* has a very broad tropism—and will infect virtually any nucleated cell type, what is observed during murine infection are predominantly infected monocytes and macrophages. In contrast to this, it is known that HFF cells begin to lose many of their cellular intrinsic defense mechanisms against intracellular pathogens at relatively low passage numbers, which makes them further unsuitable when performing *in vitro* assays. One potential way of addressing this would be to perform egress assays in a context that more accurately represents what we do see from *in vivo* studies—namely by choosing immune cell lines to replace HFFs, and performing these assays in inflammatory conditions that mimic the strong inflammatory immune response characteristic of *Toxoplasma gondii* infection. Interferon gamma is a key cytokine controlling the response⁸⁹; therefore it would be more appealing to perform egress assays in the presence of this molecule and in cell types that are able to appropriately respond to IFN gamma stimulation. Supporting this in terms of the importance to TgLCAT, it has been shown that human LPLA2 is preferentially active on oxidized substrates⁸⁷. If this is true for the *Toxoplasma* homologue then it could be that TgLCAT

is more active in oxidized environments, i.e. activated macrophages or monocytes, and studying egress in these cells would elicit a stronger phenotype.

Secondly, current methods of studying egress rely largely on inducing intracellular parasites to egress in response to pharmacologics that activate intracellular calcium signaling in the parasite. This is done largely by necessity since egress is a very dynamic process, and when allowed to occur naturally, this process typically occurs asynchronously around 48 – 60 hours post-infection. Induction is normally done with zaprinast, a phosphodiesterase inhibitor, or calcium ionophores such as ionomycin or A23187. One pitfall present in the use of all of these agents, is the off-target effects in the host cell, and their physiological relevance. Zaprinast, for instance, was originally developed by Pfizer as a drug to treat erectile dysfunction; it was abandoned due to the high number of off target effects when tested on mammalian cells⁹⁰. So while it is very effective in eliciting a calcium response from the parasite, it is also hitting a number of phosphodiesterases within the host cell, with unknown consequences. The same can also be said of the calcium ionophores—these compounds work by allowing calcium to freely cross cell membranes in a non-specific manner. Again these compounds elicit a strong response from the parasite, but certainly impact the host cell as well by liberating calcium stores from the ER, lysosomes, and mitochondria, for instance.

While off-target effects are a cause for concern when using any type of drug, it makes it especially difficult to tease apart subtle phenotypes when studying egress, because it is difficult to determine how much of the phenotype is coming from the parasite vs. the host. For example, host calpain proteases are known to play a positive role in facilitating egress of *T. gondii*. These proteases are activated by free calcium,

and are therefore likely to be activated during egress stimulation with calcium ionophores. This clouds the interpretation of the results, especially in a system studying host:pathogen interactions, because presumably any phenotype observed in egress could be due to either the parasites response, or to an altered host calpain response.

What we do know about egress *in vivo*, is that egress can be stimulated by the interaction of an infected cell with an immune cell ³¹. For example, CD8 T-cell interaction with an infected cell can be a trigger that induces parasite egress. This is presumably a way for the parasite to sense that its environment is under imminent threat, and respond appropriately. There are at least two mechanisms which describe this event, signaling through Fas ligand ³², or perforin attack³¹. A potentially improved method of studying egress *in vitro* would replace pharmacological induction of egress with a biological method. Infected cells, either transformed to express the appropriate receptors or chosen for endogenous expression of these molecules, could either be stimulated by addition of the recombinant ligands or addition of activated immune cells that express these molecules. This manner of induction would be a more appropriate approach to studying egress, because it would mimic the actual events that stimulate egress during toxoplasmosis as accurately as possible, and furthermore this would induce a response that utilizes relevant signaling pathways and would presumably elicit responses that are of a natural level. Treating monolayers in this manner may be more akin to a surgeon precisely using their scalpel vs. hitting the infected cells with a sledgehammer of calcium ionophore that robustly and indiscriminately exerts responses in both the host and parasite.

Finally, we must question the traditional method of allowing parasites to replicate within HFFs for ~30 hours before inducing egress. This is done partly out of tradition, and partly because at these later timepoints the parasites respond better to all stimulants—and in this context egress from the wild type parasites is very robust. Unfortunately, existing *in vivo* and *ex vivo* data suggests that during toxoplasmosis, parasites undergo rapid cycles of invasion and egress, on the order of a few hours ($T_{1/2} = \sim 3\text{hours}$)³¹.

Traditionally egress was thought to be a passive event, as parasite growth and replication within a host cell eventually strained the host cell to the point of physical rupture. While we now know that this is not the case, it is likely that allowing parasite to replicate and form “large” vacuoles of 32+ parasites, puts a mechanical strain on the host cell to unknown effect. This is especially apparent while attempting to study “natural egress” *in vitro*—which is typically done by trying to observe egress of vacuoles at nearly 60 hours post infection. It is unlikely that during the inflammatory stages of toxoplasmosis, the parasites never are within a single cell for that amount of time. Furthermore in cell types like monocytes and macrophages this amount of replication would be impossible as there is not the physical space necessary for 32 parasites to reside within—unlike the comparatively large HFF cells. Instead of performing egress assays at 30 hours (or later) post infection, it would seem to be far more relevant to perform these studies on cells that have only been infected for a few hours, in order to more closely mimic conditions seen during *in vivo* infection.

Traditionally egress has been a difficult event to study, in large part due to how little is known about the process overall, and due to it’s dynamic nature. Currently these

assays are done in a manner that works and is convenient, but one must question if perhaps these methods are too stimulating. This may explain why many genetic screens are failing to pick up loss of function in genes that have more mild phenotypes when compared to PLP1 or parasite motility. The development of a more fine-tuned approach would allow future work to uncover phenotypes associated with genes that have a minor impact. Additionally, these methods would allow for a more detailed mechanistic role of how these factors behave in different environmental contexts, i.e. Fas vs. Perforin attack, INF gamma vs. LPS, and even early vs. late time points. We must also consider that if further optimization and fine-tuning of *in vitro* methods to study egress still does not uncover additional effectors in this process, then either PLP1 and parasite motility are sufficient for parasite egress, or egress is not as crucial to the tachyzoite life cycle as originally thought. Which brings about another very interesting question: is egress crucial in any of the remaining parasite life stages?

REFERENCES

1. Carruthers V, Boothroyd JC. Pulling together: an integrated model of *Toxoplasma* cell invasion. *Curr Opin Microbiol.* 2007;10(1):83-89. doi: S1369-5274(06)00100-7 [pii]; 10.1016/j.mib.2006.06.017 [doi].
2. Shen B, Buguliskis JS, Lee TD, Sibley LD. Functional analysis of rhomboid proteases during *Toxoplasma* invasion. *MBio.* 2014;5(5):e01795-14. doi: 10.1128/mBio.01795-14 [doi].
3. Alexander DL, Mital J, Ward GE, Bradley P, Boothroyd JC. *Identification of the moving junction complex of Toxoplasma gondii: a collaboration between distinct secretory organelles.* *PLoS Pathog.* 2005;1(2):e17.
4. Morisaki JH, Heuser JE, Sibley LD. Invasion of *Toxoplasma gondii* occurs by active penetration of the host cell. *J Cell Sci.* 1995;108:2457-2464.
5. Bargieri D, Lagal V, Andenmatten N, Tardieux I, Meissner M, Ménard R. Host Cell Invasion by Apicomplexan Parasites: The Junction Conundrum. *PLoS Pathog.* 2014 Sep 18;10(9):e1004273. doi: 10.1371/journal.ppat.1004273. eCollection 2014 Sep.

6. Martin AM, Liu T, Lynn BC, Sinai AP. The *Toxoplasma gondii* parasitophorous vacuole membrane: transactions across the border. *J Eukaryot Microbiol.* 2007;54(1):25-28. doi: 10.1111/j.1550-7408.2006.00230.x.
7. Sinai AP. Biogenesis of and activities at the *Toxoplasma gondii* parasitophorous vacuole membrane. *Subcell Biochem.* 2008;47:155-164.
8. Chandramohanadas R, Davis PH, Beiting DP, et al. Apicomplexan parasites co-opt host calpains to facilitate their escape from infected cells. *Science.* 2009;324(5928):794-797. doi: 10.1126/science.1171085; 10.1126/science.1171085.
9. Millholland MG, Mishra S, Dupont CD, et al. A host GPCR signaling network required for the cytolysis of infected cells facilitates release of apicomplexan parasites. *Cell Host Microbe.* 2013;13(1):15-28. doi: 10.1016/j.chom.2012.12.001; 10.1016/j.chom.2012.12.001.
10. Kafsack BF, Pena JD, Coppens I, Ravindran S, Boothroyd JC, Carruthers VB. Rapid membrane disruption by a perforin-like protein facilitates parasite exit from host cells. *Science.* 2009;323(5913):530-533. doi: 10.1126/science.1165740.
11. Roiko MS, Carruthers VB. Functional Dissection of *Toxoplasma gondii* Perforin-like Protein 1 Reveals a Dual Domain Mode of Membrane Binding for Cytolysis and Parasite

Egress. *J Biol Chem*. 2013;288(12):8712-8725. doi: 10.1074/jbc.M113.450932; 10.1074/jbc.M113.450932.

12. Salmon BL, Oksman A, Goldberg DE. Malaria parasite exit from the host erythrocyte: a two-step process requiring extraerythrocytic proteolysis. *Proc Natl Acad Sci U S A*. 2001;98(1):271-276. doi: 10.1073/pnas.011413198.

13. Yeoh S, O'Donnell RA, Koussis K, et al. Subcellular discharge of a serine protease mediates release of invasive malaria parasites from host erythrocytes. *Cell*. 2007;131(6):1072-1083. doi: 10.1016/j.cell.2007.10.049.

14. Alam A, Chauhan VS. Inhibitory potential of prodomain of Plasmodium falciparum protease serine repeat antigen 5 for asexual blood stages of parasite. *PLoS One*. 2012;7(1):e30452. doi: 10.1371/journal.pone.0030452; 10.1371/journal.pone.0030452.

15. Arastu-Kapur S, Ponder EL, Fonovic UP, et al. Identification of proteases that regulate erythrocyte rupture by the malaria parasite Plasmodium falciparum. *Nat Chem Biol*. 2008;4(3):203-213. doi: 10.1038/nchembio.70.

16. Deu E, Leyva MJ, Albrow VE, Rice MJ, Ellman JA, Bogoy M. Functional studies of Plasmodium falciparum dipeptidyl aminopeptidase I using small molecule inhibitors and active site probes. *Chem Biol*. 2010;17(8):808-819. doi: 10.1016/j.chembiol.2010.06.007; 10.1016/j.chembiol.2010.06.007.

17. Lavine MD, Arrizabalaga G. Exit from host cells by the pathogenic parasite *Toxoplasma gondii* does not require motility. *Eukaryot Cell*. 2008;7(1):131-140. doi: 10.1128/EC.00301-07.
18. Egarter S, Andenmatten N, Jackson AJ, et al. The *Toxoplasma* Acto-MyoA Motor Complex Is Important but Not Essential for Gliding Motility and Host Cell Invasion. *PLoS One*. 2014;9(3):e91819. doi: 10.1371/journal.pone.0091819; 10.1371/journal.pone.0091819.
19. Farrell A, Thirugnanam S, Lorestani A, et al. A DOC2 protein identified by mutational profiling is essential for apicomplexan parasite exocytosis. *Science*. 2012;335(6065):218-221. doi: 10.1126/science.1210829; 10.1126/science.1210829.
20. Carruthers VB, Sibley LD. Mobilization of intracellular calcium stimulates microneme discharge in *Toxoplasma gondii*. *Mol Microbiol*. 1999;31(2):421-428.
21. Pace DA, McKnight CA, Liu J, Jimenez V, Moreno SN. Calcium entry in *Toxoplasma gondii* and its enhancing effect of invasion-linked traits. *J Biol Chem*. 2014;289(28):19637-19647. doi: 10.1074/jbc.M114.565390 [doi].
22. Fang J, Marchesini N, Moreno SN. A *Toxoplasma gondii* phosphoinositide phospholipase C (TgPI-PLC) with high affinity for phosphatidylinositol. *Biochem J*. 2006;394(Pt 2):417-425. doi: BJ20051393 [pii].

23. Lovett JL, Marchesini N, Moreno SN, Sibley LD. *Toxoplasma gondii* microneme secretion involves intracellular Ca^{2+} release from inositol 1,4,5-triphosphate (IP(3))/ryanodine-sensitive stores. *J Biol Chem*. 2002;277(29):25870-6.
24. Chini EN, Nagamune K, Wetzel DM, Sibley LD. Evidence that the cADPR signalling pathway controls calcium-mediated microneme secretion in *Toxoplasma gondii*. *Biochem J*. 2005;389(Pt 2):269-277. doi: 10.1042/BJ20041971.
25. Lourido S, Shuman J, Zhang C, Shokat KM, Hui R, Sibley LD. Calcium-dependent protein kinase 1 is an essential regulator of exocytosis in *Toxoplasma*. *Nature*. 2010;465(7296):359-362. doi: 10.1038/nature09022.
26. McCoy JM, Whitehead L, van Dooren GG, Tonkin CJ. TgCDPK3 regulates calcium-dependent egress of *Toxoplasma gondii* from host cells. *PLoS Pathog*. 2012;8(12):e1003066. doi: 10.1371/journal.ppat.1003066; 10.1371/journal.ppat.1003066.
27. Lourido S, Tang K, Sibley LD. Distinct signalling pathways control *Toxoplasma* egress and host-cell invasion. *EMBO J*. 2012;31(24):4524-4534. doi: 10.1038/emboj.2012.299; 10.1038/emboj.2012.299.
28. Garrison E, Treeck M, Ehret E, et al. A forward genetic screen reveals that calcium-dependent protein kinase 3 regulates egress in *Toxoplasma*. *PLoS Pathog*.

2012;8(11):e1003049. doi: 10.1371/journal.ppat.1003049;
10.1371/journal.ppat.1003049.

29. Fruth IA, Arrizabalaga G. Toxoplasma gondii: induction of egress by the potassium ionophore nigericin. *Int J Parasitol.* 2007;37(14):1559-1567. doi: 10.1016/j.ijpara.2007.05.010.

30. Moudy R, Manning TJ, Beckers CJ. The loss of cytoplasmic potassium upon host cell breakdown triggers egress of *Toxoplasma gondii*. *J Biol Chem.* 2001;276(44):41492-41501.

31. Tomita T, Yamada T, Weiss LM, Orlofsky A. Externally Triggered Egress Is the Major Fate of *Toxoplasma gondii* during Acute Infection. *J Immunol.* 2009. doi: 10.4049/jimmunol.0900516.

32. Persson EK, Agnarson AM, Lambert H, et al. Death receptor ligation or exposure to perforin trigger rapid egress of the intracellular parasite *Toxoplasma gondii*. *J Immunol.* 2007;179(12):8357-8365.

33. Nagamune K, Hicks LM, Fux B, Brossier F, Chini EN, Sibley LD. Abscissic acid controls calcium-dependent egress and development in *Toxoplasma gondii*. *Nature.* 2008;451(7175):207-210. doi: 10.1038/nature06478.

34. Bhanot P, Schauer K, Coppens I, Nussenzweig V. A surface phospholipase is involved in the migration of plasmodium sporozoites through cells. *J Biol Chem*. 2005;280(8):6752-6760. doi: 10.1074/jbc.M411465200.
35. Pszenny V, Ehrenman K, Romano JD, et al. A Lipolytic Lecithin:Cholesterol Acyltransferase Secreted by Toxoplasma Facilitates Parasite Replication and Egress. *J Biol Chem*. 2016;291(8):3725-3746. doi: 10.1074/jbc.M115.671974 [doi].
36. Hybiske K, Stephens RS. Exit strategies of intracellular pathogens. *Nat Rev Microbiol*. 2008;6(2):99-110. doi: 10.1038/nrmicro1821.
37. Smith GA, Marquis H, Jones S, Johnston NC, Portnoy DA, Goldfine H. The two distinct phospholipases C of *Listeria monocytogenes* have overlapping roles in escape from a vacuole and cell-to-cell spread. *Infect Immun*. 1995;63(11):4231-4237.
38. Shimuta K, Ohnishi M, Iyoda S, Gotoh N, Koizumi N, Watanabe H. The hemolytic and cytolytic activities of *Serratia marcescens* phospholipase A (PhlA) depend on lysophospholipid production by PhlA. *BMC Microbiol*. 2009;9:261-2180-9-261. doi: 10.1186/1471-2180-9-261 [doi].
39. Roiko MS, Svezhova N, Carruthers VB. Acidification Activates *Toxoplasma gondii* Motility and Egress by Enhancing Protein Secretion and Cytolytic Activity. *PLoS Pathog*. 2014;10(11):e1004488. doi: 10.1371/journal.ppat.1004488 [doi].

40. Abe A, Shayman JA. The role of negatively charged lipids in lysosomal phospholipase A2 function. *J Lipid Res.* 2009;50(10):2027-2035. doi: 10.1194/jlr.M900008-JLR200 [doi].
41. Hill DE, Chirukandoth S, Dubey JP. Biology and epidemiology of *Toxoplasma gondii* in man and animals. *Anim Health Res Rev.* 2005;6(1):41-61. doi: S1466252305000034 [pii].
42. Guo M, Dubey JP, Hill D, et al. Prevalence and risk factors for *Toxoplasma gondii* infection in meat animals and meat products destined for human consumption. *J Food Prot.* 2015;78(2):457-476. doi: 10.4315/0362-028X.JFP-14-328 [doi].
43. Pirofski LA, Casadevall A. The damage-response framework of microbial pathogenesis and infectious diseases. *Adv Exp Med Biol.* 2008;635:135-146. doi: 10.1007/978-0-387-09550-9_11 [doi].
44. Hunter C, Remington J. Immunopathogenesis of toxoplasmic encephalitis. *J Infect Dis.* 1994;170:1057-1067.
45. Sabin A. Toxoplasmic encephalitis in children. *JAMA.* 1941;116:801-907.
46. Jones JL, Lopez A, Wilson M, Schulkin J, Gibbs R. Congenital toxoplasmosis: a review. *Obstet Gynecol Surv.* 2001;56(5):296-305.

47. Montaner JSG, Le T, Hogg R, et al. The changing spectrum of AIDS index diseases in Canada. *AIDS*. 1994;8:693-696.
48. Luft BJ, Remington JS. Toxoplasmic encephalitis in AIDS. *Clin Infect Dis*. 1992;15:211-222.
49. Strittmatter C, Lang W, Wiestler OD, Kleihues P. The changing pattern of human immunodeficiency virus-associated cerebral toxoplasmosis: a study of 46 postmortem cases. *Acta Neuropathol*. 1992;83(5):475-481.
50. Suss-Toby E, Zimmerberg J, Ward GE. *Toxoplasma* invasion: the parasitophorous vacuole is formed from host cell plasma membrane and pinches off via a fission pore. *Proc Natl Acad Sci USA*. 1996;93:8413-8418.
51. Arrizabalaga G, Ruiz F, Moreno S, Boothroyd JC. Ionophore-resistant mutant of *Toxoplasma gondii* reveals involvement of a sodium/hydrogen exchanger in calcium regulation. *J Cell Biol*. 2004;165(5):653-662. doi: 10.1083/jcb.200309097.
52. Burda PC, Roelli MA, Schaffner M, Khan SM, Janse CJ, Heussler VT. A *Plasmodium* phospholipase is involved in disruption of the liver stage parasitophorous vacuole membrane. *PLoS Pathog*. 2015;11(3):e1004760. doi: 10.1371/journal.ppat.1004760 [doi].

53. Huynh MH, Carruthers VB. Tagging of endogenous genes in a *Toxoplasma gondii* strain lacking Ku80. *Eukaryot Cell*. 2009;8(4):530-539. doi: 10.1128/EC.00358-08.
54. Shen B, Brown KM, Lee TD, Sibley LD. Efficient gene disruption in diverse strains of *Toxoplasma gondii* using CRISPR/CAS9. *MBio*. 2014;5(3):e01114-14. doi: 10.1128/mBio.01114-14 [doi].
55. Stommel EW, Ely KH, Schwartzman JD, Kasper LH. *Toxoplasma gondii*: dithiol-induced Ca^{2+} flux causes egress of parasites from the parasitophorous vacuole. *Exp Parasitol*. 1997;87(2):88-97.
56. Ruecker A, Shea M, Hackett F, et al. Proteolytic activation of the essential parasitophorous vacuole cysteine protease SERA6 accompanies malaria parasite egress from its host erythrocyte. *J Biol Chem*. 2012;287(45):37949-37963. doi: 10.1074/jbc.M112.400820; 10.1074/jbc.M112.400820.
57. Wirth CC, Glushakova S, Scheuermayer M, et al. Perforin-like protein PPLP2 permeabilizes the red blood cell membrane during egress of *Plasmodium falciparum* gametocytes. *Cell Microbiol*. 2014;16(5):709-733. doi: 10.1111/cmi.12288 [doi].
58. Garg S, Agarwal S, Kumar S, Shams Yazdani S, Chitnis CE, Singh S. Calcium-dependent permeabilization of erythrocytes by a perforin-like protein during egress of

malaria parasites. *Nat Commun.* 2013;4:1736. doi: 10.1038/ncomms2725; 10.1038/ncomms2725.

59. Deligianni E, Morgan RN, Bertuccini L, et al. A perforin-like protein mediates disruption of the erythrocyte membrane during egress of *Plasmodium berghei* male gametocytes. *Cell Microbiol.* 2013. doi: 10.1111/cmi.12131; 10.1111/cmi.12131.

60. Sidik SM, Huet D, Ganesan SM, et al. A Genome-wide CRISPR Screen in *Toxoplasma* Identifies Essential Apicomplexan Genes. *Cell.* 2016;166(6):1423-1435.e12. doi: 10.1016/j.cell.2016.08.019 [doi].

61. Brown KM, Lourido S, Sibley LD. Serum Albumin Stimulates Protein Kinase G-dependent Microneme Secretion in *Toxoplasma gondii*. *J Biol Chem.* 2016;291(18):9554-9565. doi: 10.1074/jbc.M115.700518 [doi].

62. Shuttleworth TJ. Arachidonic acid, ARC channels, and Orai proteins. *Cell Calcium.* 2009;45(6):602-610. doi: 10.1016/j.ceca.2009.02.001 [doi].

63. Watanabe H, Vriens J, Prenen J, Droogmans G, Voets T, Nilius B. Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature.* 2003;424(6947):434-438. doi: 10.1038/nature01807 [doi].

64. Hsiao CH, Luisa Hiller N, Haldar K, Knoll LJ. A HT/PEXEL motif in *Toxoplasma* dense granule proteins is a signal for protein cleavage but not export into the host cell. *Traffic*. 2013;14(5):519-531. doi: 10.1111/tra.12049 [doi].
65. Mollay C, Kreil G. Enhancement of bee venom phospholipase A2 activity by melittin, direct lytic factor from cobra venom and polymyxin B. *FEBS Lett*. 1974;46(1-2):141-144. doi: [http://dx.doi.org/10.1016/0014-5793\(74\)80354-6](http://dx.doi.org/10.1016/0014-5793(74)80354-6).
66. LaFavers KA, Marquez-Nogueras KM, Coppens I, Moreno SNJ, Arrizabalaga G. A novel dense granule protein, GRA41, regulates timing of egress and calcium sensitivity in *Toxoplasma gondii*. *Cell Microbiol*. 2017;19(9):10.1111/cmi.12749. Epub 2017 May 17. doi: 10.1111/cmi.12749 [doi].
67. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature*. 2005;434(7030):214-217. doi: nature03342 [pii].
68. Marti M, Good RT, Rug M, Knuepfer E, Cowman AF. Targeting malaria virulence and remodeling proteins to the host erythrocyte. *Science*. 2004;306(5703):1930-1933. doi: 306/5703/1930 [pii].
69. Cooke BM, Mohandas N, Coppel RL. Malaria and the red blood cell membrane. *Semin Hematol*. 2004;41(2):173-188. doi: S0037196304000058 [pii].

70. Hiller NL, Bhattacharjee S, van Ooij C, et al. A host-targeting signal in virulence proteins reveals a secretome in malarial infection. *Science*. 2004;306(5703):1934-1937. doi: 306/5703/1934 [pii].
71. Boddey JA, Hodder AN, Gunther S, et al. An aspartyl protease directs malaria effector proteins to the host cell. *Nature*. 2010;463(7281):627-631. doi: 10.1038/nature08728 [doi].
72. de Koning-Ward TF, Gilson PR, Boddey JA, et al. A newly discovered protein export machine in malaria parasites. *Nature*. 2009;459(7249):945-949. doi: 10.1038/nature08104 [doi].
73. Beck JR, Muralidharan V, Oksman A, Goldberg DE. PTEX component HSP101 mediates export of diverse malaria effectors into host erythrocytes. *Nature*. 2014;511(7511):592-595. doi: 10.1038/nature13574 [doi].
74. Jones TC, Hirsch JG. The interaction between *Toxoplasma gondii* and mammalian cells. II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. *J Exp Med*. 1972;136(5):1173-94.
75. Michelin A, Bittame A, Bordat Y, et al. GRA12, a *Toxoplasma* dense granule protein associated with the intravacuolar membranous nanotubular network. *Int J Parasitol*. 2009;39(3):299-306. doi: 10.1016/j.ijpara.2008.07.011 [doi].

76. Rosowski EE, Lu D, Julien L, et al. Strain-specific activation of the NF-kappaB pathway by GRA15, a novel *Toxoplasma gondii* dense granule protein. *J Exp Med*. 2011;208(1):195-212. doi: 10.1084/jem.20100717 [doi].
77. Bougdour A, Durandau E, Brenier-Pinchart MP, et al. Host cell subversion by *Toxoplasma* GRA16, an exported dense granule protein that targets the host cell nucleus and alters gene expression. *Cell Host Microbe*. 2013;13(4):489-500. doi: 10.1016/j.chom.2013.03.002 [doi].
78. Curt-Varesano A, Braun L, Ranquet C, Hakimi MA, Bougdour A. The aspartyl protease TgASP5 mediates the export of the *Toxoplasma* GRA16 and GRA24 effectors into host cells. *Cell Microbiol*. 2016;18(2):151-167. doi: 10.1111/cmi.12498 [doi].
79. Coffey MJ, Sleebs BE, Uboldi AD, et al. An aspartyl protease defines a novel pathway for export of *Toxoplasma* proteins into the host cell. *Elife*. 2015;4:10.7554/eLife.10809. doi: 10.7554/eLife.10809 [doi].
80. Hammoudi PM, Jacot D, Mueller C, et al. Fundamental Roles of the Golgi-Associated *Toxoplasma* Aspartyl Protease, ASP5, at the Host-Parasite Interface. *PLoS Pathog*. 2015;11(10):e1005211. doi: 10.1371/journal.ppat.1005211 [doi].
81. Gold DA, Kaplan AD, Lis A, et al. The *Toxoplasma* Dense Granule Proteins GRA17 and GRA23 Mediate the Movement of Small Molecules between the Host and the

Parasitophorous Vacuole. *Cell Host Microbe*. 2015;17(5):642-652. doi: 10.1016/j.chom.2015.04.003 [doi].

82. Schaloske RH, Dennis EA. The phospholipase A2 superfamily and its group numbering system. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*. 2006;1761(11):1246-1259. doi: <http://dx.doi.org.proxy.lib.umich.edu/10.1016/j.bbalip.2006.07.011>.

83. Hagelueken G, Clarke BR, Huang H, et al. A coiled-coil domain acts as a molecular ruler to regulate O-antigen chain length in lipopolysaccharide. *Nat Struct Mol Biol*. 2015;22(1):50-56. doi: 10.1038/nsmb.2935 [doi].

84. Laliberte J, Carruthers VB. Toxoplasma gondii toxolysin 4 is an extensively processed putative metalloproteinase secreted from micronemes. *Mol Biochem Parasitol*. 2011;177(1):49-56. doi: 10.1016/j.molbiopara.2011.01.009.

85. Miller SA, Binder EM, Blackman MJ, V C, Kim K. A conserved subtilisin-like protein TgSUB1 in microneme organelles of *Toxoplasma gondii*. *J Biol Chem*. 2001;276(48):45341-8.

86. Brown WC, DelProposto J, Rubin JR, Lamiman K, Carless J, Smith JL. New ligation-independent cloning vectors compatible with a high-throughput platform for

parallel construct expression evaluation using baculovirus-infected insect cells. *Protein Expr Purif*. 2011;77(1):34-45. doi: 10.1016/j.pep.2011.01.007 [doi].

87. Abe A, Hiraoka M, Ohguro H, Tesmer JJ, Shayman JA. Preferential hydrolysis of truncated oxidized glycerophospholipids by lysosomal phospholipase A2. *J Lipid Res*. 2017;58(2):339-349. doi: 10.1194/jlr.M070730 [doi].

88. Schanne FA, Kane AB, Young EE, Farber JL. Calcium dependence of toxic cell death: a final common pathway. *Science*. 1979;206(4419):700-702.

89. Denkers EY. T lymphocyte-dependent effector mechanisms of immunity to *Toxoplasma gondii*. *Microbes Infect*. 1999;1(9):699-708.

90. Ballard SA, Gingell CJ, Tang K, Turner LA, Price ME, Naylor AM. Effects of sildenafil on the relaxation of human corpus cavernosum tissue in vitro and on the activities of cyclic nucleotide phosphodiesterase isozymes. *J Urol*. 1998;159(6):2164-2171. doi: S0022-5347(01)63299-3 [pii].

91. Glukhova A, Hinkovska-Galcheva V, Kelly R, Abe A, Shayman JA, Tesmer JJ. Structure and function of lysosomal phospholipase A2 and lecithin:cholesterol acyltransferase. *Nat Commun*. 2015;6:6250. doi: 10.1038/ncomms7250 [doi].

92. Coffee M, Dagley L, Kapp E, Infusini G, Boddey J, Webb A, Tonkin C. Aspartyl protease 5 matures virulence factors found at the host-parasite interface in *Toxoplasma gondii*. bioRxiv. Feb 26, 2018. doi: <http://dx.doi.org/10.1101/271676>.
93. Schaloske, R. H. & Dennis, E. A. The phospholipase A2 superfamily and its group numbering system. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1761, 1246-1259 (2006).
94. Ollis, D. L. *et al.* The alpha/beta hydrolase fold. *Protein Eng.* 5, 197-211 (1992).
95. Arpigny, J. L. & Jaeger, K. E. Bacterial lipolytic enzymes: classification and properties. *Biochem. J.* 343 Pt 1, 177-183 (1999).
96. Dennis, E. A., Cao, J., Hsu, Y. H., Magrioti, V. & Kokotos, G. Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. *Chem. Rev.* 111, 6130-6185 (2011).
97. Jaeger, K. E. Extracellular enzymes of *Pseudomonas aeruginosa* as virulence factors. *Immun. Infekt.* 22, 177-180 (1994).
98. Cajal, Y., Svendsen, A., Girona, V., Patkar, S. A. & Alsina, M. A. Interfacial control of lid opening in *Thermomyces lanuginosa* lipase. *Biochemistry* 39, 413-423 (2000).

99. Jaeger, K. E. Extracellular enzymes of *Pseudomonas aeruginosa* as virulence factors. *Immun. Infekt.* 22, 177-180 (1994).